

Hormonal Regulation of Long Chain Fatty Uptake by Adipocytes and Studies of the FATP Gene Family

by

David J. Hirsch

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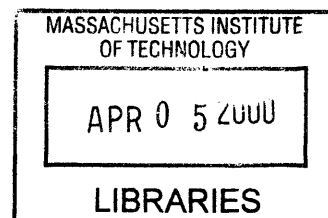
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Submitted to the Department of Biology on December 30, 1999 in Partial Fulfillment
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ABSTRACT

Long chain fatty acids (LCFA) are an important source of energy for most organisms. Serum fatty acid (FA) levels are dynamically regulated by hormones. We show that insulin directly stimulates adipocyte fatty acid influx suggesting that the decrease in serum FA levels seen after meals is partially mediated by an insulin-stimulated increase in FA uptake by adipocytes. We also find that TNF- α directly inhibits FA uptake by 3T3-L1 adipocytes providing a physiologic link between the increased serum levels of TNF- α and FA seen in Type II diabetes. Transport of LCFA across the plasma membrane is facilitated by FATP, a plasma membrane protein that increases LCFA uptake when expressed in cultured mammalian cells (Schaffer and Lodish, 1994). We report the identification of family of evolutionarily conserved FATPs. In addition to the gene first isolated by Schaffer and Lodish, humans have five additional FATP homologues (designated FATP1-5). All of the mammalian FATPs increase fatty acid uptake when over-expressed in COS cells. FATPs are also found in such diverse organisms as *F. rubripes*, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, and *M. tuberculosis*. The function of the FATP gene family is conserved throughout evolution as the *C. elegans* and mycobacterial FATPs facilitate LCFA uptake when over-expressed in COS cells or *E. coli*, respectively. Among the mammalian FATPs, several are expressed in a highly tissue specific manner. We show that FATP4 is expressed at high levels on the apical side of mature enterocytes in the small intestine. Furthermore, reduction of FATP4 expression in primary enterocytes by anti-sense oligonucleotides inhibits FA uptake by 50% suggesting that FATP4 is the principal fatty acid transporter in enterocytes. FATP5 is expressed only in adult liver. We have isolated the FATP5 promoter and find that tissue specific expression can be recapitulated *in vitro* and requires a single GC box in conjunction with two novel ten nucleotide motifs. Additional study of the FATP gene family may provide a better understanding of the mechanisms whereby LCFAs traverse the lipid bilayer as well as yield insight into the control of energy homeostasis and its dysregulation in diseases such as diabetes and obesity.

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Apportionment of labor

Chapter 1 Introduction

Text	David Hirsch
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Chapter 2 Hormonal regulation of fatty acid uptake by adipocytes

Text and intellectual contributions	Andreas Stahl and David Hirsch
FACS plots and data	David Hirsch
Radiolabeled efflux	Andreas Stahl
Radiolabeled uptake	Andreas Stahl

Chapter 3 A family of fatty acid transporters conserved from mycobacteria to man

Text	David Hirsch
Intellectual contribution	Andreas Stahl
BLAST searches and alignment	David Hirsch
Library screening	David Hirsch and Andreas Stahl
FATP alignment	David Hirsch
FACS plots and data	David Hirsch and Andreas Stahl
Northern analysis	David Hirsch
Family tree	Andreas Stahl

Hirsch, D., Stahl, A., and Lodish, H. F. (1998). A family of fatty acid transporters conserved from mycobacterium to man. Proc Natl Acad Sci U S A 95, 8625-9.

Chapter 4 Identification of the major intestinal fatty acid transport protein

Text	Andreas Stahl
Intellectual contribution	David Hirsch
GST fusion construct and protein	David Hirsch and Mariana Kotler
<i>In situ</i> hybridization	MPI
Antibody purification and characterization	Andreas Stahl and Mariana Kotler
Immunofluorescence	Andreas Stahl and Nicki Watson
Electronmicroscopy	Andreas Stahl and Nicki Watson
Antisense and enterocyte uptake	Andreas Stahl
FATP4 stable cell line	Andreas Stahl and MPI
BODIPY competition studies	MPI

Stahl, A., Hirsch, D. J., Gimeno, R. E., Punreddy, S., Ge, P., Watson, N., Patel, S., Kotler, M., Raimondi, A., Tartaglia, L. A., and Lodish, H. F. (1999). Identification of the major intestinal fatty acid transport protein. *Mol Cell* 4, 299-308.

Chapter 5 Identification and characterization of the FATP5 promoter

Text	David Hirsch
Intellectual contribution	Andreas Stahl
All figures and constructs	David Hirsch

Chapter 6 Concluding thoughts

Text	David Hirsch
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Chapter 1

Introduction

Although often neglected, obesity is arguably the single most important epidemic facing the developed world. After holding steady for about 20 years, the percentage of the United States' population classified as clinically obese almost doubled from 14.5% to 22.5% over the period from 1976 to 1994 (Taubes, 1998). The negative health consequences of obesity are significant. Severely obese people have a 40-fold greater risk of non-insulin dependent diabetes mellitus (NIDDM) than normal controls and more than 80% of NIDDM cases can be attributed to obesity (Bray, 1996). Obesity is also associated with a 160% increase in mortality from cardiovascular disease (Gillham et al., 1997). One study estimates that 34,000 of the 55,000 deaths caused by diabetes and 77,000 of the 400,000 deaths from coronary heart disease each year are attributable to obesity (Bray, 1996). Obesity is also a risk factor for cirrhosis, gall stones, and cancer. Monetarily, obesity costs the United States between \$50 and \$70 billion a year in direct and indirect costs (Bray, 1996; Wickelgren, 1998). Furthermore, Americans spend almost \$40 billion a year on weight loss treatments (Wickelgren, 1998).

The increased prevalence of obesity is generally considered to be the result of environmental factors. Essentially, people are eating more and exercising less. The

average person in a developed country now receives about 40% of their daily calories from fat (Thomson et al., 1997). Almost all of this fat is in the form of triglyceride with oleate and palmitate as the major components. Although fat absorption is exclusively confined to the small intestine, digestion of fat begins in the mouth with lingual lipase, which can hydrolyze approximately one-third of the ingested fat. Gastric lipase released by the stomach can digest an additional 10-40% of the triglyceride with the remainder being digested by pancreatic lipase. The pancreas also secretes bile salts which help emulsify the fat and facilitate digestion. Fatty acids, bile salts, mono-acyl glycerols, and phospholipids form mixed micelles in the small intestine. These micelles diffuse to the brush border of the enterocytes which line the small intestine and the fatty acids are taken up by these cells. After absorption, the fatty acids are esterified to CoA, reformed into triglyceride, packaged into chylomicrons, and secreted into the lymph.

Recently, a promising area of obesity therapy has been the reduction of fat intake either through inhibition of fat digestion in the small intestine or the use of non-digestible fat substitutes in food. Olestra is a fat substitute in which six to eight fatty acids are esterified to a sucrose backbone (Thomson et al., 1997). Unlike fatty acids esterified to glycerol, the fatty acids in Olestra cannot be removed by lipases and consequently are not absorbed in the small intestine. The impact of Olestra on weight reduction has been limited due to restrictions on its use in food. Xenical (orlistat) is a compound developed by Hoffman-LaRoche that inhibits gastric and pancreatic lipase. In male individuals with a daily intake of 76 grams of fat per day, 80 mg of orlistat can reduce the absorption of fatty acid uptake by 36%. However, the effectiveness of orlistat is limited by several factors including the action of lingual lipase which is unaffected by the drug. Both Olestra and Xenical can cause loose stools, vitamin malabsorption and gastrointestinal distress which further limits their usefulness.

Another potential strategy for obesity therapy would be to reduce fat absorption from the mixed micelle by the enterocytes. However, for many years, absorption of fatty acids by the enterocytes was thought to be diffusion-mediated. Some investigators have challenged this notion and suggested that fatty acid uptake by enterocytes is a protein-mediated process. If so, inhibitors of these proteins would be potential anti-obesity medications. Although elucidating the mechanism of fatty acid uptake in the small intestine has immediate therapeutic relevance, the general question of how fatty acids cross the plasma membrane applies to many other tissues. In addition to the intestine, many organs such as the heart and liver possess a robust ability to take up free fatty acids from the blood. However, as with the small intestine, the mechanism whereby these tissues take up fatty acids is controversial.

Diffusion mediates fatty acid transport across the lipid bilayer.

How do fatty acids enter a cell? The answer to this question partly depends on whether fatty acids freely traverse the lipid bilayer. Several investigators have studied this issue and determined that fatty acids traverse the lipid bilayer sufficiently fast *in vitro* to account for the rates of fatty acid transport measured *in vivo*. Although the number of papers addressing this issue is voluminous, the general principles can be elucidated by examining the work of two groups in greater detail.

The first group, headed by James Hamilton, has developed a robust system to study the trans-bilayer movement of fatty acids (Hamilton, 1998; Kamp and Hamilton, 1992; Kamp et al., 1993). He defines three requisite steps for fatty acid movement across a

lipid bilayer. The first step, adsorption, is the transfer of the fatty acids from albumin, the primary carrier for free fatty acids in serum, to the lipid bilayer. The second step, transmembrane movement, is the transfer of lipids from the outside of the lipid bilayer to the inside leaflet. The final step of fatty acid transport, desorption, is the transfer of the fatty acid from the internal leaflet to the intracellular sites other than the plasma membrane. Adsorption of fatty acids to the lipid bilayer from albumin (bovine serum albumin (BSA) in this case) was studied in small unilamellar phosphatidyl choline vesicles (SUV) utilizing ^{13}C NMR. By measuring the change in the NMR signal of the ^{13}C labeled fatty acid to determine its location, Hamilton found that albumin-complexed fatty acids rapidly equilibrate between albumin and SUVs. The $t_{1/2}$ for the equilibration of octanoate and deconoate were 2 msec and 35 msec respectively and it was assumed that other fatty acids would transfer with a similarly rapid rate. To study transmembrane movement of fatty acids, Hamilton developed a novel assay using SUVs with a pH-sensitive dye trapped inside. When fatty acids were added to a preparation of these SUVs, the pH inside the vesicle rapidly decreased by approximately 0.2 pH units implying that fatty acids traverse the lipid bilayer as a protonated species. Although the pKa of fatty acids in aqueous solution is approximately 5, Hamilton's group estimated that the pKa of the fatty acids in the membrane environment of the SUV was closer to 7.5. Thus, approximately 50% of the fatty acids would be protonated at any given time and able to flip-flop from the outer to the inner leaflet of the SUV lipid bilayer (Hamilton and Cistola, 1986). Ionization of protonated fatty acids after transmembrane movement lowered the pH of the vesicle and trapped them in the inner leaflet of the SUV. Consistent with this mechanism, prior acidification of the SUV suppressed fatty acid movement and larger amounts of fatty acids added to the outside of the SUV produced greater changes in pH inside the vesicle. The rate of fatty acid transmembrane movement was very rapid. pH equilibrium in SUVs was complete within 1 msec after mixing SUVs

and the BSA-fatty acid solution. In large unilamellar vesicles (LUVs), the $t_{1/2}$ was 35 msec. In both cases, the rate of equilibration was completely independent of fatty acid chain length. The final step in fatty acid transport defined by Hamilton, desorption, requires the fatty acid to partition from the inner hemi-leaflet of the bilayer to the cytoplasm. Desorption was measured in SUVs containing fatty acids and a pH-sensitive indicator within the vesicle. In this system, a rise in pH indicated fatty acid movement out of the SUV. Using BSA as an acceptor for fatty acids from the outer leaflet of the SUV, the $t_{1/2}$ for the transfer of fatty acids from the SUVs to BSA was less than 1 second for fatty acids between 14 and 26 carbons long. Although this system required transmembrane movement of fatty acids before desorption, the rate of fatty acid movement from SUVs to BSA was much slower than the previously measured rate for transmembrane movement of fatty acids. Thus, the rate of fatty acid movement from SUVs to BSA in this assay reflects the rate of desorption and not transmembrane movement.

Studies similar to Hamilton's were conducted by Kleinfeld et al. (Kleinfeld and Storch, 1993). Their assay utilized 12-(9-anthroyloxy) stearic acid (12AS), a fluorescent fatty acid derivative, to measure fatty acid movement. Experimentally, a donor population of either SUV or LUVs loaded with 12AS was mixed with a population of recipient vesicles pre-loaded with N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylcholine (NBD-PE) which quenched 12AS fluorescence. Transfer of 12AS from the donor to recipient vesicles resulted in a decrease in fluorescence. The transfer of 12AS from donor to recipient followed a bi-exponential curve which could be broken into a slow and fast component. In contrast to Hamilton's results, the slow step in 12AS transport was transmembrane flip-flop while transfer of 12AS between the vesicle populations was the fast step. Furthermore, Kleinfeld et al. found that the rate of 12AS movement was 10-

fold slower in LUVs than SUVs suggesting that vesicle size and curvature were important parameters in fatty acid diffusion. The rate of diffusion was also dependent on the length of the fatty acid as a short chain anthroxy fatty acid (<12 carbons) transferred more than 100 times faster between lipid vesicles than the longer chain 12AS. Although 12AS could easily diffuse between artificial lipid vesicles, Kleinfeld et al. concluded that 12AS flip-flop would be too slow to accommodate the high rate of fatty acid uptake by certain tissues. Subsequent work by Kamp et al. found that 12AS flip-flop was much faster than that measured by Kleinfeld et al. and would not be rate-limiting for fatty acid transport (Kamp et al., 1995).

In order to extrapolate the results of fatty acid transfer in artificial bilayers to a more physiologically relevant system, Hamilton et al. examined fatty acid uptake by a pancreatic β -cell line (Hamilton et al., 1994). Since fatty acid transfer in an artificial lipid vesicle required the flip-flop of a protonated fatty acid, cytosolic pH should decrease after addition of exogenous fatty acids if a similar process occurred in cultured cells. Addition of 50 nmol oleate (30 μ M final concentration) to the pancreatic β -cells resulted in an acidification of 0.2 pH units after 10 minutes. The majority of the fatty acids taken up by the cell line was not esterified and oleate efflux could be stimulated by the addition of BSA to the media which rapidly reversed the acidification caused by oleate. In addition to oleate, a fatty acid dimer comprised of two long chain fatty acids coupled through a covalent bond between carbons in the fatty acid chains also caused intracellular acidification. Moreover, addition of tetradecylamine, a fatty acid analog whose ionizable group is an amine instead of a carboxylic acid, resulted in intracellular alkanization which would be predicted if the uncharged amine flip-flopped to the intracellular side and some of these molecules subsequently became protonated. Since three structurally dissimilar compounds traversed the lipid bilayer by a similar mechanism and it is unlikely that a

single protein could recognize all three structures, Hamilton et al. concluded that a plasma membrane transporter could not be responsible for fatty acid uptake *in vivo*. Similarly, Civelek et al. found that addition of 100 μ M oleate to primary adipocytes caused a rapid acidification which reached a maximum of 0.2 pH units after 5 minutes (Civelek et al., 1996). Addition of 1 μ M BSA to the adipocytes at this point reversed the pH change suggesting that the absorbed fatty acids had not been metabolized. Tetradecylamine caused intracellular alkalinization and a fatty acid dimer (the same used in the previous study) caused intracellular acidification. Although the $t_{1/2}$ for acidification of the adipocyte was much faster than that observed in pancreatic cells, the overall mechanism of fatty acid uptake in the two cell types appeared to be identical. Activators of hormone-sensitive lipase including norepinephrine, forskolin, and isoproterenol led to intracellular acidification which, in the case of isoproterenol, could be prevented by the addition of exogenous BSA. Civelek et al. hypothesized that decreases in intracellular pH reflected an increase in the cytoplasmic free fatty acid concentrations expected after treatment with lipolytic agents. On the other hand, insulin, which antagonizes lipolysis, caused an intracellular alkalinization that could be markedly attenuated by the addition of BSA to the media.

From the studies outlined above, it is clear that fatty acids can rapidly move into and out of artificial lipid vesicles without a protein to facilitate this process. The cell line data indicate that a similar mechanism of fatty acid transfer probably occurs *in vivo*. These investigators propose that fatty acid metabolism is the rate-limiting for fatty acid uptake. Noy et al. have estimated that *in vivo* myristate uptake by the liver is 20 times slower than what can be extrapolated from *in vitro* models (Noy et al., 1985). Saturation of fatty acid uptake is merely a consequence of the saturation of a metabolic pathway. Thus, can simple physicochemical properties account for the transfer of fatty acids from blood to

the tissues? Contrary to the data presented above, three lines of evidence suggest that the answer to this question is likely “no”.

First, artificial lipid vesicles may not accurately predict the behavior of real cells. The surface area to volume ratio of a SUV is 10^5 that of a normal cell (Schaffer and Lodish, 1995). Thus, movements of a small number of fatty acids could have a substantial change on intravesicular pH. Moreover, since SUVs and LUVs don't predict the behavior of each other, how can the kinetic data from these artificial lipid vesicles be extrapolated cells orders of magnitude larger? Storch et al. found that fatty acid transport in SUVs was 10 times faster than in LUVs (Storch and Kleinfeld, 1986). While the movement of fatty acids into and out of LUVs was a symmetrical process, the movement of fatty acids in SUVs was asymmetrical. Transfer of fatty acids from the inside leaflet of the SUV lipid bilayer into the inside of the vesicle was twice as fast as the movement of fatty acids from the outside leaflet to the media. Storch et al. also found that the transfer rates of long chain fatty acids (C16 or C18) between lipid vesicles was more than two orders of magnitude slower than that for short chain fatty acids (Storch and Kleinfeld, 1986). However, long chain fatty acids are taken up much more efficiently than short chain fatty acids *in vivo*.

Abumrad et al. found that ^3H -oleate uptake was 15 times greater than that of ^{14}C -octanoate in primary rat adipocytes (Abumrad et al., 1984). Similarly, Luiken et al. found that octanoate uptake into heart giant vesicles was only 1% of palmitate uptake (Luiken et al., 1999). Although proponents of diffusion would suggest that the *in vivo* data reflect differences in the metabolism of short chain versus long chain fatty acids, the large discrepancy between artificial vesicles and living cells is disconcerting.

Second, the adipocyte and pancreatic β -cell studies should be viewed with caution. Although the mechanism of fatty acid uptake in SUVs and mammalian cells appeared to be similar, the time course of cytosolic acidification in adipocytes was on the order of minutes in stark contrast to the millisecond pH changes which occur in SUVs. Furthermore, several aspects of fatty acid uptake in these cell lines were not consistent with the biology of fatty acid metabolism *in vivo*. Since greater than 95% of fatty acids taken up by adipocytes are esterified within one minute, the vast majority of fatty acids taken up by the cell after five minutes would be trapped within the adipocyte (Berk et al., 1997). Thus, the reversibility of cytosolic acidification by BSA after five minutes of fatty acid uptake observed by Hamilton et al. cannot be due to the extraction of free fatty acids from the cell. These studies also used ultra-physiologic levels of fatty acids. Whereas the *in vivo* concentration of free fatty acids in the blood is less than 100 nM, Hamilton et al. used 30 μ M and Civelek et al. used 100 μ M oleate. Also, most of the fatty acid uptake data were generated in the absence of albumin which is normally present at 300 μ M in serum. When these groups did include BSA, the fatty acid:BSA ratio was 8:1 whereas the physiologic range is 0.5-0.8:1 in fasted mice (Fitscher et al., 1996). The physiologic relevance of the pH changes induced by lipolytic agents in adipocytes is also difficult to extrapolate *in vivo*. Since addition of only 1 μ M BSA to the media greatly attenuated cytosolic acidification, hormonally induced pH changes would be unlikely to occur *in vivo* where the concentration of albumin is hundreds of times higher.

Third, when studied in intact tissues, fatty acid transport has hallmarks of a protein facilitated process including saturability, competability and specificity. Such evidence, some of which is reviewed below, has been gathered in a variety of experimental systems and tissues including heart, liver, intestine, fat, and muscle.

Protein-mediated fatty acid transport across the lipid bilayer

The intestine has a large capacity for nutrient absorption. Amino acids and sugars have defined mechanisms for absorption and the molecular basis for these processes is known in great detail. In contrast, the mechanism of fatty acid uptake is somewhat enigmatic. S. Chow and D. Hollander studied fatty acid uptake in everted intestinal sacs (Chow and Hollander, 1978). To simulate the normal presentation of fatty acids in the small intestine, they used taurocholate as a carrier for the fatty acid. They found that ^3H -arachidonic acid uptake was biphasic. Between 5 and 150 μM , ^3H -arachidonic acid uptake was saturable while at concentrations between 150 μM and 8.3 mM ^3H -arachidonic acid uptake was non-saturable. Although, everted sacs from either the proximal or distal small intestine absorbed arachidonate equally well at high concentrations, sacs from the proximal small intestine had a three-fold greater ability to take up arachidonate at a low arachidonate concentration. At low concentrations of ^3H -arachidonate, addition of long chain fatty acids such as oleate, linoleate, and linolenate to the everted sacs inhibited ^3H -arachidonic acid uptake. However, butyrate, a short chain fatty acid, and unrelated substances transported by other systems such as glutamate, lysine, leucine, and dextrose had no effect on ^3H -arachidonic acid uptake. Thus, arachidonate uptake in the small intestine occurs *via* two distinct mechanisms. While high concentrations of arachidonate are transported by a non-saturable mechanism, lower concentrations of arachidonate are taken up through a saturable, competitive, and specific process indicative of protein-mediated facilitated transport. In a related study, they found that ^{14}C -linoleate uptake had properties similar to that of arachidonate (Chow and Hollander, 1979). Between 42 and 1260 μM linoleate uptake was saturable while

linoleate uptake between 2.5 and 4.2 mM was a linear function of fatty acid concentration. Similar to the data for ^3H -arachidonate, other long chain fatty acids inhibited ^{14}C -linoleate uptake. Addition of unlabeled arachidonate increased the K_m without changing the V_{\max} for ^{14}C -linoleate uptake suggesting that arachidonate was a competitive inhibitor of linoleate uptake. In addition to everted intestinal sacs, they found similar results for ^{14}C -linoleate uptake *in vivo* using cannulated segments of jejunum in intact, unanesthetized rats (1979; Chow and Hollander, 1978). Following these studies on isolated sections of intestine, Gore and Hoinard studied linoleic acid uptake in isolated intestinal cells (Gore and Hoinard, 1993). Presented to the enterocytes in taurocholate micelles, ^{14}C -linoleic acid uptake had a K_m of 382 nM and a V_{\max} of 11.37 nmol / mg protein / min. Uptake at 4°C was essentially zero and a 10-fold molar excess of unlabeled oleate, linoleate, and other long chain fatty acids reduced ^{14}C -linoleic acid uptake by more than 90% while addition of glucose or alanine had no effect. Trotter et al. studied fatty acid uptake in the Caco-2 human intestinal cell line (Trotter et al., 1996). At 7-12 days post-confluence, oleate and palmitate uptake in Caco-2 cells was saturable and high affinity. While oleate and palmitate uptake in Caco-2 cells displayed Michaelis-Menten kinetics, ^3H -octanoate uptake was non-saturable. Furthermore, ^3H -oleate uptake could be inhibited by excess oleate but not octanoate, and ^3H -palmitate uptake could be inhibited by excess palmitate but not octanoate. However, neither excess octanoate nor palmitate reduced ^3H -octanoate uptake. Thus, specific uptake of fatty acids in Caco-2 cells is restricted to long chain fatty acids. The results of Gore and Hoinard in isolated enterocytes and Trotter et al. in an intestinal cell line further support the notion that fatty acid uptake in the small intestine takes place through a specific, saturable, and competitive mechanism.

Instead of the taurocholate micelles found in the lumen of the small intestine, the primary buffer for circulating fatty acids is albumin. At physiologic levels of albumin (between 300 and 600 μM), the majority of fatty acids in the blood are bound to albumin and the free fatty acid concentration is usually less than 100 nM. Experimentally, free fatty acid concentrations can be altered by either changing the amount of a labeled fatty acid in the presence of a fixed albumin concentration or changing the albumin concentration in the presence of a fixed amount of fatty acid. Then, using equations derived by Spector et al., the free fatty acid concentration can be calculated for any given concentration of fatty acid and albumin (Spector et al., 1969). To a first approximation, fatty acid:BSA ratios of 2:1 or less are likely to be physiologically relevant. Unless noted otherwise, concentrations of fatty acids cited below refer to the free fatty acid concentration.

The liver is a central organ for fat metabolism. The fenestrated endothelium affords easy access of the hepatocytes to the fatty acid bound albumin in blood. Fatty acid uptake by the liver is very robust and first pass hepatic clearance of fatty acids from the blood has been estimated to be higher than 50%. An elegant study of isolated hepatocytes quantitated fatty acid uptake by measuring the release of $^{14}\text{CO}_2$ after the addition of ^{14}C -palmitate to the media (Mahadevan and Sauer, 1974). Addition of trypsin to the hepatocytes reduced $^{14}\text{CO}_2$ release by 67% but had no effect on $^{14}\text{CO}_2$ production when ^{14}C -octanoate was added to the hepatocytes. Since octanoate oxidation was unaffected, Mahadevan et al. hypothesized that trypsin decreased $^{14}\text{CO}_2$ production from palmitate by inhibiting long chain fatty acid uptake in hepatocytes. Sorretino et al. studied oleate uptake in intact livers and isolated hepatocytes (Sorrentino et al., 1996). By titrating in different amounts of ^3H -oleate in the presence of 600 μM BSA to vary the free ^3H -oleate concentration, they determined that the K_m for oleate uptake in hepatocyte suspensions was 83 nM. Addition of unlabeled palmitate to the cell suspension increased the K_m for

^3H -oleate without affecting the V_{\max} indicating a competitive mechanism of inhibition. They then measured ^3H -oleate uptake in whole livers using a multiple indicator dilution format that included ^3H -oleate, ^{125}I -albumin, and ^{14}C -sucrose. Addition of a 50-fold molar excess of unlabeled palmitate specifically reduced ^3H -oleate uptake by more than 10-fold but had no effect on the other radiolabeled substances. Stremmel et al. further characterized oleate uptake in isolated hepatocytes (Stremmel et al., 1986). They found that oleate uptake in hepatocytes was critically dependent on the unbound oleate concentration. Titrating in different amounts of oleate in the presence of a fixed amount of albumin or varying the amount of albumin in the presence of a fixed oleate concentration to manipulate the free oleate concentration yielded identical K_m and V_{\max} values for oleate uptake. When fatty acid uptake was plotted as a function of the free oleate, the kinetics of oleate uptake in both experimental set-ups was identical. Oleate uptake was specific as anions such as sulfobromophthalein, taurocholate, and cholic acid did not inhibit fatty acid uptake. These results were expanded upon by Stump et al. (Stump et al., 1992). At an oleate:BSA ratio of 2 or less, fatty acid uptake was saturable with a K_m of 153 nM. Fatty acid uptake using oleate:BSA ratios greater than 3 was entirely due to a non-saturable process and had no definable K_m . Since the physiologic fatty acid:albumin ratio is much less than 2.5, the authors concluded that greater than 90% of the total hepatic oleate uptake was due to a saturable process. Stremmel and Berk also provided evidence of protein-mediated fatty acid transport by studying fatty acid efflux in hepatocytes (Stremmel and Berk, 1986). In the absence of exogenous glucose, 89% of the oleate taken up by hepatocytes after 30 seconds was non-esterified. Addition of BSA to the stop solution stimulated fatty acid efflux from the hepatocytes in a biphasic manner. After an initial rapid removal of 55% of the cell-associated radioactivity, an additional 35% of the sequestered fatty acid was gradually lost over the next five minutes. When they added phloretin, a non-specific, cell-impermeable inhibitor of many

protein-mediated transport processes, to the BSA stop solution, the initial, rapid loss of cell-associated oleate was unchanged while the slower efflux process was completely abolished. They suspected that the initial loss of cell-associated radioactivity reflected the removal of fatty acids non-specifically bound to the outside of the cell while the slower phase represented efflux of internalized fatty acids through a protein-mediated process. To support this hypothesis, they examined oleate uptake in hepatocytes at 4°C when protein-mediated transport processes would be expected to be inhibited. Although oleate uptake was reduced by 50% at 4°C, greater than 90% of the cell-associated oleate could be instantly removed by BSA. Moreover, phloretin had no inhibitory effect on the ability of BSA to sequester oleate under these conditions.

Skeletal and cardiac muscle have a high capacity to metabolize fatty acids. In fact, fatty acids are the preferred energy source of the heart, which is able to extract 40-70% of the unesterified fatty acids in the blood on a single pass (Glatz et al., 1997). Luiken et al. found that palmitate uptake in isolated cardiac myocytes exhibited characteristics of a protein-mediated process (Luiken et al., 1997). Palmitate uptake was saturable and high affinity with a K_m of 9 nM. Since palmitate oxidation and esterification exhibited the same saturation characteristics as palmitate uptake, they hypothesized that palmitate uptake was rate-limiting for these processes. Consistent with this notion, phloretin reduced palmitate uptake and oxidation by more than 80%. In subsequent studies using heart giant vesicles, Luiken et al. found that palmitate uptake had a K_m of 10 nM and a V_{max} of 2.7 pmol / mg protein / second (Luiken et al., 1999). Palmitate uptake in heart and skeletal giant vesicles had the same K_m , but the V_{max} in heart was 8 times that of skeletal muscle. ^3H -palmitate uptake in heart giant vesicles was competitive by palmitate and oleate but not octanoate or glucose. Furthermore, ^3H -octanoate uptake was less than 1% that of palmitate uptake in heart and only 2-3% that of palmitate in muscle. C. Elsing

et al. studied fatty acid uptake in skeletal myocytes using confocal laser microscopy and 12-NBD-stearate, a fluorescent fatty acid derivative (Elsing et al., 1998). Although the K_m of 12-NBD stearate was only 366 nM, 12-NBD-hexanoate uptake was not detectable.

Since adipose tissue is the primary storehouse of fat in the body, if protein-mediated transport of fatty acids existed, one would certainly expect to find such a system in adipocytes. Abumrad et al. found that initial rates of ^{14}C -oleate uptake by primary adipocytes was a saturable process (Abumrad et al., 1981). In the absence of exogenous glucose, the oleate taken up by the adipocytes remained unesterified and slowly leaked out of the cell after the addition of cold buffer. Addition of BSA to the stop solution increased the rate of oleate efflux 100-fold. Similar to the results of Stremmel et al. in liver, Abumrad et al. found that phloretin inhibited both influx and efflux of oleate in adipocytes. In a subsequent study they found that radiolabeled stearate, oleate, linoleate, and palmitate were all taken up by adipocytes in a saturable manner (Abumrad et al., 1984). The K_m 's for stearate and oleate were 0.16 and 0.06 μM respectively and both fatty acids had similar V_{\max} values. Laurate, a 10-carbon fatty acid, was also taken up by adipocytes in a saturable manner but had a K_m of 1.5 μM . The K_m for ^{14}C -stearate uptake was the same as the stearate K_i for inhibition of ^3H -oleate uptake (and vice versa for oleate inhibition of ^{14}C -stearate uptake) suggesting that stearate and oleate uptake proceeded through a common pathway. The degree of inhibition of ^{14}C -linoleate uptake by oleate, linoleate, palmitate and laurate could also be predicted by their K_m 's. In contrast, ^{14}C -octanoate uptake was 20 times lower than that for oleate and was not competable by oleate, nor was octanoate able to inhibit ^3H -oleate uptake. Furthermore, phloretin inhibited oleate, stearate, and laurate uptake but not octanoate uptake. In addition to fatty acid chain length, the carboxyl group was also important for specificity as the methyl ester of oleate did not inhibit ^{14}C -oleate uptake but alpha-bromopalmitate

did. Finally, as discussed later, a variety of cell surface modifying reagents potentially inhibited fatty acid uptake into adipocytes.

There are many more examples of saturable and competitive uptake of fatty acids in various systems. However, skeptics of fatty acid uptake argue that saturability, competitiveness and specificity are not proof of a facilitated transport mechanism. They might still argue that transmembrane movement of fatty acids is diffusional and fatty acid uptake is limited by metabolism. Thus, proteins which enhance fatty acid uptake do so by enhancing the metabolism of fatty acids thereby maintaining a concentration gradient of free fatty acids from out to in.

Acyl-CoA synthases enhance fatty acid uptake

In support of this hypothesis, fatty acid acyl-CoA synthases (FACS) have been proposed to enhance fatty acid transport in a variety of systems. In order to be used for phospholipid synthesis, storage, or oxidation, fatty acids must first be esterified to coenzyme A (CoA). A set of well characterized, evolutionarily conserved enzymes catalyzes this ATP-requiring reaction. The first suggestion that FACS was necessary for transport of fatty acids came from studies in *E. coli* mutants that were unable to utilize long chain fatty acids as an exogenous carbon source (Black and DiRusso, 1994). The prefix *fad* (fatty acid degradative) was attached to these genes and many of them encoded proteins involved in beta-oxidation. However, two of these genes, *fadL* and *fadD*, were crucial for fatty acid uptake from the media. The product of the *fadL* gene was shown to be an integral outer membrane protein and hypothesized to facilitate fatty acid transport across this layer. The other gene required for fatty acid uptake, *fadD*, was an acyl-CoA

synthase. Furthermore, depletion of either cellular ATP or CoA, two necessary cofactors for FACS activity also reduced fatty acid uptake. Thus, in *E. coli*, esterification of fatty acids to CoA is essential for fatty acid uptake. Subsequent work by Schaffer and Lodish found that over-expression of a mammalian FACS in Cos cells increased fatty acid uptake (Schaffer and Lodish, 1994). Consistent with an important role in fatty acid uptake, FACS was recently shown to be an integral plasma membrane protein in 3T3-L1 adipocytes and associated with Glut 4-containing vesicles in primary adipocytes (Gargiulo et al., 1999; Sleeman et al., 1998). Although FACS is essential for fatty acid uptake in *E. coli* and over-expression of FACS in mammalian cells can stimulate fatty acid uptake, this does not preclude the existence of additional proteins involved in fatty acid uptake from either cooperating with FACS or acting upstream of it. Indeed, the existence of *fadD* in *E. coli* proves this point. Similarly, Gargiulo et al. have recently shown that FACS and a putative fatty acid transporter can synergistically increase fatty acid uptake in mammalian cells (Gargiulo et al., 1999).

Fatty acid binding proteins enhance fatty acid uptake

Instead of esterifying fatty acids to CoA, other groups have suggested that sequestration of intracellular free fatty acids by fatty acid binding protein's (FABPs) would facilitate fatty acid transport. FABPs were first identified in 1972 by R. Ockner et al. (Ockner et al., 1972). They made cytosolic extracts from small intestine or liver, incubated them with ³H-oleate, and fractionated the mixture over a Sephadex G-75 column. They found two major peaks of radioactivity eluting from the column. The first peak was albumin and the second peak was a novel, low molecular weight protein designated fatty acid binding protein (FABP). 95% of the bound radioactivity could be extracted from the

FABP peak and thin layer chromatography of the extracted radioactivity showed it to be unmodified oleate. FABP avidly bound many long chain fatty acids including linoleate, oleate, and palmitate but did not bind octanoate, a medium chain fatty acid. FABP was also identified in cytosolic extracts from liver, heart, fat, and kidney but was not found in plasma or red cells. Although initially identified as a single binding activity present in a variety of tissues, FABP activity turned out to be mediated by a family of homologous proteins. Cloning of the FABPs revealed that they were part of a large superfamily of proteins called lipocalins. The lipocalin gene family includes homologous proteins which bind hydrophobic substances including retinol, retinoic acid, or bile acids (Schaap et al., 1998). Although these proteins share only 14-66% homology at the amino acid level, crystal structures of several family members revealed a common tertiary structure (Flower, 1996). The FABP gene family contains at least 9 mammalian genes, many of which are expressed in a highly tissue-specific manner (Glatz et al., 1997; Ockner, 1990). FABPs are also expressed at very high levels in some tissues. H-FABP (heart-FABP) and L-FABP (liver-FABP) account for 3% of the cytoplasmic protein in rat heart and liver respectively (Schaap et al., 1998; Schroeder et al., 1993). Binding of fatty acids to FABPs is also relatively specific. While H-FABP binds both saturated and unsaturated fatty acids with nanomolar affinity, it has a low affinity for acyl-CoA esters, acyl-carnitine esters, or long chain fatty acid alcohols. One of the first suggestions that FABPs might be involved in fatty acid uptake was from a study by David Waggoner and David Bernlohr (Waggoner and Bernlohr, 1990). They found that addition of a photo-activatable fatty acid derivative, ^{125}I -Iodo-4-azido-N-hexadecylsalicylamide (^{125}I -AHS), to 3T3-L1 adipocytes resulted in the specific labeling of a 14 kDa cytoplasmic protein. Using specific antibodies, the cross-linked protein was identified as adipocyte-lipid binding protein (ALBP or A-FABP). ALBP was not labeled in undifferentiated 3T3-L1 fibroblasts which also had a 10-fold lower ability to take up oleate than the differentiated

3T3-L1 adipocytes. From these data, they concluded that ALBP might play a central role in fatty acid uptake in the adipocyte. Further support for this idea came from Sha et al. who found that over-expression of ALBP in Chinese hamster ovary cells (CHO cells) increased oleate uptake by 1.5-2-fold (Sha et al., 1993). On the other hand, CHO cells over-expressing a mutant ALBP unable to bind fatty acids had oleate uptake levels comparable to wild-type CHO cells. Additional groups have also found evidence implicating FABPs in fatty acid uptake. Over-expression of L-FABP in L cell fibroblasts increased NBD-stearate and cis-parinaric uptake by 1.7 and 1.2-fold respectively (Murphy, 1998; Schroeder et al., 1993). Similarly, over-expression of I-FABP in ES cells increased the rate of NBD-stearate uptake 1.7-fold (Atshaves et al., 1998). Instead of over-expressing FABPs in cell lines to stimulate fatty acid uptake, Wolfrum et al. tried to inhibit fatty acid uptake by lowering FABP expression. (Wolfrum et al., 1999). Stable expression of a L-FABP anti-sense cDNA in HepG2 cells (a liver cell line) lowered the concentration of L-FABP protein from 0.2% to 0.03% of cytosolic protein. The six-fold decrease in L-FABP expression was accompanied by a 66% reduction in oleate uptake. Additionally, using data from other clones, they determined that the degree of inhibition of L-FABP expression correlated with the decrease in fatty acid uptake.

Probably the best experimental evidence linking FABP and fatty acid uptake comes from studies of knockout mice. In nullizygous H-FABP mice, cardiac uptake of ^{125}I -BMIPP, a non-metabolizable fatty acid analog, was reduced by greater than 80% (Binas et al., 1999). To compensate for the decreased availability of fatty acids for energy production, cardiac uptake of ^{14}C -deoxy-glucose was increased 5-fold. ^{125}I -BMIPP uptake in liver, which does not express H-FABP, was unaffected in the knockout mouse. Although H-FABP knockout mice were viable and fertile, 3-month-old mice had a 38-fold increase in atrial natriuretic peptide (ANP), a marker for cardiac hypertrophy. By one year of age,

H-FABP mice developed overt histological changes similar to cardiac hypertrophy. Compared to wild-type mice, 3-month-old H-FABP knockout mice were also exercise intolerant. A-FABP (adipocyte FABP) has also been knocked out (Hotamisligil et al., 1996). Although Hotamisligil et al. did not measure adipocyte fatty acid uptake, A-FABP knockout mice gained weight at the same rate as normal mice suggesting that adipocyte fatty acid uptake was normal. However, the analysis of the A-FABP knockout mouse was confounded by a 20-fold upregulation of K-FABP (keratinocyte FABP) in the adipocytes of knockout mice. Interestingly, when A-FABP knockout mice were placed on a high fat diet, they gained more weight than either heterozygous or wild-type mice but did not become insulin-resistant.

However, while several studies have shown that over-expression of FABPs increases fatty acid uptake, other studies have been unable to demonstrate such an effect. Transfection of L6 myoblasts with either H-FABP or A-FABP had no effect on palmitate uptake, and transient transfection of Cos cells with A-FABP also had no effect on fatty acid uptake (Prinsen and Veerkamp, 1998; Schaffer and Lodish, 1994). Although Murphy et al. found that L-FABP was able to stimulate fatty acid uptake in L-cells, similar levels of I-FABP (intestinal-FABP) had no effect (Murphy, 1998). Consistent with this result, Prows et al. and Holehouse et al. also found that I-FABP over-expression did not increase oleate uptake (Holehouse et al., 1998; Prows et al., 1995). In contrast to the results Murphy et al. reported for L-FABP over-expression in L-cells, Fitscher et al. did not see an increase in fatty acid uptake when L-FABP mRNA was injected into *Xenopus* oocytes (Fitscher et al., 1995).

So, if FABPs don't facilitate fatty acid transport by sequestering them in the cell, what is their function? Several pieces of evidence support the notion that FABPs facilitate the

movement of fatty acids through the cytoplasm. Consistent with this hypothesis, more than 95% of the cytosolic fatty acids are bound to FABP and inhibition of fatty acid binding to FABPs greatly reduces the amount of fatty acids found in the cytoplasm (Luxon, 1996). In a subsequent study, Luxon et al. found that removal of FABPs from the cell dramatically inhibits the intracellular diffusion of fatty acids (Luxon and Milliano, 1997). Using fluorescence recovery after photobleaching (FRAP), they determined that the diffusion coefficient (D_{eff}) for NBD-stearate in HepG2 cells was $6.7 \times 10^{-9} \text{ cm}^2 / \text{sec}$. Permeabilization of HepG2 cells with streptolysin-O to remove cytoplasmic binding proteins reduced the D_{eff} to $0.39 \times 10^{-9} \text{ cm}^2 / \text{sec}$ which closely approximates the value for lateral diffusion of fatty acids within membranes. Incubation of the permeabilized cells with 150 μM FABP increased the D_{eff} five-fold. Interestingly, addition of 150 μM BSA to the permeabilized cells restored the D_{eff} of NBD-stearate to that of unpermeabilized levels. Although, FABP and BSA are very different proteins, both bind fatty acids with high affinity. However, since cells don't have high levels of albumin in the cytoplasm, FABPs are probably the proteins responsible for facilitating fatty acid movement through the cytoplasm *in vivo*. Finally and perhaps most directly, over-expression of FABPs increases the D_{eff} 's for fatty acids (Atshaves et al., 1998; Murphy, 1998). In addition to the experimental evidence outlined above, several teleological reasons suggest that the lateral movement of membrane-bound fatty acids does not contribute significantly to fatty acid flux through the cell (Luxon, 1996). First, the movement of membranes through the cell is orders of magnitude slower than the movement of fatty acids. Second, lateral diffusion of fatty acids in the membrane is more than 10 times slower than the diffusion of fatty acids in the cytoplasm. Third, the highly convoluted nature of membranes would greatly increase the distance a fatty acid would need to travel to get to a particular organelle.

By facilitating the movement of fatty acids through the cell, FABPs may modulate lipid metabolism. Baier et al. studied the effect of an I-FABP polymorphism in which substitution of threonine for alanine⁵⁴ results in a two-fold higher affinity for fatty acids *in vitro* (Baier et al., 1996). They measured fatty acid transport in Caco-2 monolayers by adding radiolabeled fatty acids to the apical side of the monolayer and assaying radioactivity secreted by the cells basolaterally. Caco-2 cell lines over-expressing Thr⁵⁴ I-FABP transported twice as much palmitate or oleate as cells expressing Ala⁵⁴ I-FABP. As a control, glucose transport across the monolayer was the same in both cell lines. However, the fatty acids released basolaterally were in the form of triglyceride and the cell-associated radioactivity was the same in both cell lines. Thus, Thr⁵⁴ I-FABP accelerated the formation and secretion of triglyceride by Caco-2 cells but had no effect on fatty acid uptake. A similar link between FABPs and lipid metabolism was demonstrated in cell lines over-expressing L-FABP (Murphy et al., 1996). Although L-FABP increased ³H-oleate uptake by 1.8-fold, incorporation of ³H-oleate into cholesterol esters was increased 11-fold and incorporation of radioactivity into choline glycerophospholipids and phosphatidylserine was increased 13 and 6-fold respectively. These stimulatory effects were specific as the rate of oleate incorporation into other phospholipids was unchanged.

However, if FABPs do not transport fatty acids, why does the H-FABP knockout have a 80% decrease in fatty acid uptake? One idea would build on the notion that FABPs are required for the efficient metabolism of fatty acids. Consistent with this hypothesis, Vork et al. calculated that expression of h-FABP in the heart results in a 17-fold increase in the flux of fatty acids from the sarcolemma to the mitochondria (Vork et al., 1993). In the absence of H-FABP, mitochondrial fatty acid oxidation would be reduced leading to a

buildup of fatty acids within the cell that would subsequently inhibit the uptake of additional fatty acids from the blood.

Plasma membrane proteins mediate fatty acid uptake

FABP and FACS clearly play important roles in lipid metabolism and, either directly or indirectly, are linked to fatty acid transport. Thus, is there any reason to invoke the existence of additional proteins required for fatty acid transport?

Several lines of evidence argue that FABP and FACS are not solely responsible for fatty acid uptake. First, studies using non-metabolizable fatty acids have shown saturable and competent uptake (Abumrad et al., 1981; Elsing et al., 1998; Fraser et al., 1997; Stremmel et al., 1986). Second, fatty acid uptake is independent of metabolism (Abumrad et al., 1991; Abumrad et al., 1981; Berk et al., 1997; Luiken et al., 1999; Storch et al., 1991; Stremmel and Berk, 1986; Trotter et al., 1996). Third, cell-impermeable protein modifying reagents such as phloretin and stilbene derivatives inhibit fatty acid uptake (Abumrad et al., 1984; Ibrahimi et al., 1996). Fourth, treatment of intact cells with proteases also inhibits fatty acid uptake (Abumrad et al., 1984; Fraser et al., 1997; Guthmann et al., 1999; Luiken et al., 1999; Luiken et al., 1997; Schwieterman et al., 1988; Sorrentino et al., 1988; Stremmel and Berk, 1986). Since FABP and FACS are intracellular proteins, the last two results clearly indicate that additional plasma membrane proteins are required for fatty acid uptake. In fact, no less than six candidate fatty acid transporters have been identified. Each of these will be discussed in greater detail below.

FABPpm

Stremmel et al. identified the first putative plasma membrane fatty acid transporter (Stremmel et al., 1985). They found high affinity, saturable, heat-denaturable oleate binding sites in purified rat liver plasma membranes (LPM) and attempted to purify the activity with an oleate-agarose affinity column. Following extensive washing of the LPMs applied to the oleate agarose column, 8 M urea eluted a single 40 kDa protein which they designated LPM fatty acid binding protein (LPM-FABP or FABPpm). Similar to Ockner's results with intracellular FABPs more than a decade earlier, Stremmel et al. found that FABPpm co-migrated with radiolabeled oleate, palmitate, linoleate, and arachidonate on a gel filtration column. Binding of long chain fatty acids was specific as FABPpm did not bind the cholesterol ester of oleate, phosphatidylcholine containing oleate in the 2 position, or other hydrophobic substances including bilirubin, taurocholate, and sulfobromophthalein. Antibodies to FABPpm stained the plasma membrane of hepatocytes and inhibited ^3H -oleate binding to LPMs. FABPpm antibodies also inhibited oleate, palmitate, stearate, and arachidonate uptake into hepatocytes but had no effect on sulfobromophthalein, cholate, or taurocholate uptake (Stremmel and Theilmann, 1986). The reduction in fatty acid uptake was due to a decrease in the V_{max} suggesting that the antibody was a non-competitive inhibitor of fatty acid uptake. Similar studies by other investigators have shown that FABPpm antibodies can inhibit fatty acid uptake into heart giant vesicles (Luiken et al., 1999), placental choriocarcinoma cells (Campbell et al., 1997), adipocytes (Schwieterman et al., 1988; Zhou et al., 1992), and cardiomyocytes (Sorrentino et al., 1988) to cite a few examples.

However, the story was enormously complicated when many lines of evidence converged to suggest that FABPpm was identical to the mitochondrial isoform of aspartate aminotransferase (mAspAT). Purified FABPpm and mAspAT had identical molecular weights, isoelectric points, tryptic digest patterns, amino acid composition, identity for the N-terminal 35 amino acids, and antibodies to each protein recognized the other (Berk et al., 1990; Stump et al., 1993). mAspAT bound to an oleate agarose column and purified FABPpm had aspartate aminotransferase activity. Antibodies to mAspAT inhibited oleate but not glucose or octanoate uptake by adipocytes and pre-absorption of the mAspAT antibody with purified FABPpm abolished the ability of the antibody to inhibit fatty acid uptake (Zhou et al., 1992). Further proof of the identity of FABPpm and mAspAT was provided by Isola et al. who transfected 3T3 fibroblasts with a cDNA for mAspAT (Isola et al., 1995). Stable cell lines derived from this construct had increased levels of oleate but not glucose or octanoate uptake. Furthermore, unlike most transporters, FABPpm was not an integral membrane protein. Although immunofluorescence in unpermeabilized cells showed FABPpm staining on the plasma membrane, 80% of the FABPpm in a plasma membrane preparation could be extracted from the membranes without detergent (Stump et al., 1993).

Thus, a large amount of data support two seemingly irreconcilable facts. First, FABPpm is a fatty acid transporter. Second, FABPpm is identical to mAspAT. A possible solution to this conundrum was suggested by Zhou et al. (Zhou et al., 1992). Although undifferentiated 3T3-L1 fibroblasts and differentiated adipocytes both expressed high levels of intracellular FABPpm, plasma membrane staining of FABPpm was only detected in adipocytes. These results were confirmed by western analysis of subcellular fractions prepared from fibroblasts and adipocytes. Furthermore, in the mAspAT stable cell lines generated by Isola et al., FABPpm plasma staining was only detected in cells

over-expressing mAspAT although western blots clearly showed that control cells expressed lower levels of FABPpm (Isola et al., 1995). Perhaps FABPpm only functions as a fatty acid transporter when targeted to the plasma membrane by some as yet undefined mechanism. In order to understand the physiologic role of FABPpm in modulating fatty acid uptake, future studies must focus on uncovering the specific mechanism whereby FABPpm facilitates fatty acid transport.

FAT (CD36)

Inhibition of adipocyte fatty acid uptake by non-permeable protein modifying reagents implies the existence of plasma membrane fatty acid transporters. In addition to proteases, Abumrad et al. found that DIDS and SITS, potent inhibitors of many anion transporters, reduced fatty acid uptake into adipocytes by 90% (Abumrad et al., 1984). Since DIDS is a relatively non-specific inhibitor, Harmon et al. synthesized a sulfosuccinimidyl ester of oleate (SSO) as a potentially more selective protein modifying reagent (Harmon et al., 1991). SSO dose dependently inhibited oleate, linoleate, and stearate uptake in adipocytes by 65% while leaving glucose transport unaffected. In contrast to SSO, pretreatment of adipocytes with either oleate or free N-hydroxysulfosuccinimide had no effect on subsequent measurements of fatty acid transport. Furthermore, sulfosuccinimidyl-propionate (SSP), a short chain fatty acid derivative, had no effect on oleate uptake while another long chain fatty acid derivative, sulfosuccinimidyl myristate (SSM), inhibited stearate and linoleate influx into adipocytes. Electrophoresis of plasma membranes from cells treated with radiolabeled DIDS, SSO, or SSM revealed a common 85 kDa protein labeled by all three compounds. SSO labeled only the 85 kDa band, DIDS and SSM also labeled a 75 kDa band, and

DIDS alone labeled an additional 95 kDa band. Using a similar approach, Tanaka and Kawamura found that a sulfosuccinimidyl ester of palmitate (SSP) reduced fatty acid uptake in rat heart by 25% (Tanaka and Kawamura, 1995). ^3H -SSP also crosslinked a single 85 kDa band in rat heart plasma membranes. Guthmann et al. recently demonstrated that SSO inhibited palmitate uptake by 75% in type II pneumocytes (Guthmann et al., 1999).

Hamilton et al. purified the 85 kDa band labeled by SSO in adipocytes and N-terminal sequence revealed it to be identical to CD36 and PAS IV (Note: CD36 and PAS IV are the same protein) (Harmon and Abumrad, 1993). Polyclonal antibodies to PAS IV recognized a 85kDa band from adipocyte plasma membranes and a monoclonal antibody to CD36 had previously been shown to strongly label the plasma membrane of rat adipocytes. Using degenerate oligonucleotides based on the N-terminal peptide sequence, Abumrad et al. cloned a cDNA for the 85 kDa band from an adipocyte library (Abumrad et al., 1993). Termed fatty acid translocase (FAT), the nucleotide sequence was 75% identical to human CD36^a. Although the nucleotide sequence predicted a protein of only 472 amino acids, extensive glycosylation caused it to run much larger than predicted by the primary amino acid sequence on SDS-page. Hydropathy analysis predicted two transmembrane domains, one at the N-terminus and the other at the C-terminus. CD36 mRNA was expressed at high levels in heart, spleen, intestine, and skeletal muscle. Low levels were present in testis and CD36 mRNA was absent in liver and kidney. Consistent with a role in dietary fatty acid uptake, Poirer et al. found that CD36 was abundantly expressed in jejunum and duodenum (Poirier et al., 1996). In the

^a Consistent with a recently published review on fatty acid transporters written by Abumrad et al., FAT will be referred to as CD36.

villi of the jejunum, CD36 protein was highest in villus tip cells and absent from crypt cells which parallels the distribution of fatty acid uptake in the villus. Furthermore, within the enterocyte, the CD36 protein was confined to the brush border, which is the site for nutrient absorption. CD36 expression in the small intestine was also regulated. High-fat diets rich in long chain fatty acids induced expression of CD36 in small intestine while a medium chain high fat diet only weakly enhanced CD36 expression. Abumrad et al. also found that differentiation of 3T3-442A, Ob1771, or BFC-1 cells from fibroblasts to adipocytes was accompanied by a dramatic increase in CD36 mRNA. The time course of CD36 mRNA induction in BFC-1 cells correlated with increases in oleate uptake. Over-expression of CD36 in Ob17PY fibroblasts increased oleate uptake 4-fold while 2 deoxy-glucose uptake was unaltered (Ibrahimi et al., 1996). In contrast to the low levels of oleate uptake in untransfected cells, fatty acid uptake in CD36 over-expressing cells was saturable, high affinity ($K_m=4$ nM), and sensitive to phloretin. CD36 has also been shown to directly bind fatty acids *in vitro*. Using CD36 purified from rat adipocytes, Baillie et al. found that oleic, stearic, arachidonic, palmitic, and linoleic acids reversibly bound CD36 with high affinity (Baillie et al., 1996). They further estimated that up to 3 moles of fatty acid could be bound per mole of CD36. Interestingly, they noted that amino acids 127-279 of CD36 had 75% homology with muscle-fatty acid binding protein (M-FABP) suggesting a common fatty acid binding motif. Similar to experiments in which a FABPpm antibody could inhibit fatty acid uptake, Dutta-Roy et al. found that a CD36 monoclonal antibody inhibited arachidonate and oleate uptake in platelets by 57% and 84% respectively (Dutta-Roy et al., 1996).

Genetic manipulation of CD36 expression in mice has provided further evidence of a role in lipid metabolism. CD36 knockout mice had a significant decrease in adipocyte fatty acid uptake at a fatty acid:BSA ratio of 0.5 (Febbraio et al., 1999). These mice also had

elevated serum levels of cholesterol, triglycerides, and free fatty acids but have lower levels of plasma glucose. In another mouse model, Ibrahimi et al. generated transgenic mice over-expressing CD36 specifically in muscle (Ibrahimi et al., 1999). Muscle from transgenic mice had a greatly enhanced ability to increase palmitate oxidation in response to contraction. Although the authors did not directly measure fatty acid uptake in isolated myocytes, serum levels of free fatty acids and triglycerides were reduced in transgenic mice. Further support of a link between CD36 levels and fatty acid oxidation was provided by Bonen et al. who found that chronic stimulation of skeletal muscle increased palmitate uptake and CD36 expression (Bonen et al., 1999).

Perhaps the most intriguing link between CD36 and fatty acid transport is from humans deficient in CD36 expression. CD36 deficiency was first identified in a thrombocytopenic patient in 1989 (Nozaki et al., 1999). Since then two types of CD36 deficiency have been identified. In Type I disease, neither platelets nor monocytes express CD36 while in Type II disease, monocytes express CD36 but platelets do not. Heart fatty acid utilization can be measured *in vivo* using ^{123}I -15-(p-iodophenyl)-3-(R,S)-methyl pentadecanoic acid (^{123}I -BMIPP). ^{123}I -BMIPP is an iodinated beta-methyl-branched fatty acid that can be transported into cells and converted to ^{123}I -BMIPP-CoA. Using ^{123}I -BMIPP, several studies have shown that patients with type I CD36 deficiency have a complete lack of cardiac ^{123}I -BMIPP accumulation (Hwang et al., 1998; Nozaki et al., 1999; Watanabe et al., 1998). Interestingly patients with this deficiency have a four-fold increase in cardiac glucose uptake suggesting a compensatory mechanism for decreased fatty acid uptake (Fukuchi et al., 1999). Unfortunately, Febbraio et al. did not measure cardiac fatty acid uptake in the CD36 knockout which would have established a causal link between CD36 and cardiac fatty acid uptake (Febbraio et al., 1999).

Although abundant data support the hypothesis that CD36 is a fatty acid transporter, several lines of evidence argue against this idea. First, CD36 is not expressed in liver, which has a tremendous ability to take up fatty acids from circulation (Stremmel, 1989). In fact, patients with type I CD36 deficiency show normal levels of ^{123}I -BMIPP accumulation in their liver. Second, CD36 is expressed at very high levels in the spleen, which is not known to extensively take up fatty acids from circulation (Becker and Bruce, 1985). Third, CD36 probably has no role in the absorption of dietary fatty acids. If CD36 were responsible for fatty acid uptake in the gut, as the localization studies implied, knockout mice should have been unable to absorb dietary fat and no such defect was reported. Fourth, and perhaps most significantly, CD36 does not specifically bind long chain fatty acids. Although CD36 can bind fatty acids, it has also been shown to bind many other substances in a competent, saturable manner including oxidized LDL (Ox-LDL), acetylated LDL, maleylated BSA, collagen, anionic phospholipids, Plasmodium falciparum infected erythrocytes, HDL, LDL, and VLDL (Acton et al., 1994; Calvo et al., 1998; Oquendo et al., 1989; Rigotti et al., 1995). Although structurally unrelated, many of these substances will inhibit the binding of each other, suggesting that they interact with a common binding site on CD36. For example, phosphatidylinositol, phosphatidylserine, and maleylated BSA will inhibit ^{125}I -acetylated LDL binding to Cos cells transiently transfected with CD36 (Acton et al., 1994; Rigotti et al., 1995). Specific binding of lipoproteins to CD36 may be mediated by the lipid portion of lipoproteins as the binding of HDL to CD36 does not require ApoE (Calvo et al., 1998). Furthermore, Ox-LDL binding to CD36 can be competed by oleate and delipidated Ox-LDL cannot bind CD36 (Nicholson et al., 1995). CD36 may be responsible for binding Ox-LDL *in vivo* as peritoneal macrophages from CD36 knockout mice are defective in their ability to bind and internalize Ox-LDL (Febbraio et al., 1999).

The exact role of CD36 in lipid metabolism is obscured by its ability to bind to a plethora of ligands. Perhaps a lesson can be drawn from studies of SR-BI, a homologue of CD36. Although SR-BI binds a similar spectrum of ligands as CD36 *in vitro*, Acton et al. have shown that SR-BI likely plays a key role in HDL metabolism *in vivo* (Sue Acton, Science, 271, 518-20, 1996). Thus, although SR-BI has a broad *in vitro* specificity, its physiologic role is perhaps much more narrowly defined. Hopefully, further studies of CD36 will similarly clarify its physiologic function^b.

FATP

FAT and FABPpm were first identified by their ability to be cross-linked by fatty acid derivatives. These proteins were then cloned and subsequent studies performed to show a role in fatty acid transport. In contrast to this approach, Schaffer and Lodish went the other direction (Schaffer and Lodish, 1994). They used a FACS-based expression cloning strategy to identify cDNAs which increased the uptake of 4,4-difluoro-5-methyl-4-bora-3a,4a, diaza-3-indacene-3-dodecanoic acid (BODIPY-FA), a fluorescent fatty acid derivative, when transfected into Cos cells. After several rounds of enrichment, they identified two cDNAs from an adipocyte library which increased fatty acid uptake when over-expressed in Cos cells. One of the cDNAs was a long chain acyl-CoA synthase discussed previously. The other cDNA was a novel gene which they designated fatty acid transport protein (FATP). The FATP cDNA predicted a protein of 646 amino acids with a N-terminal signal sequence and multiple transmembrane domains. The only

^b Although initial data suggested that mutations in FAT are responsible for the SHR phenotype (hypertension, hyperinsulinemia, glucose intolerance, hypertriglyceridemia, and visceral obesity), a recent study by Gotoda et al. has shown otherwise (Aitman et al., 1999; Gotoda et al., 1999).

significant homology to other proteins was a stretch of 11 amino acids found in many proteins that catalyze reactions involving carboxylic acids. FATP was induced upon differentiation of 3T3-L1 fibroblasts into adipocytes and was present at high levels in skeletal muscle, heart, brain, fat, and kidney. Interestingly, FATP was absent from liver and small intestine, two organs known to take up large amounts of fatty acids. Cell lines stably over-expressing FATP had increased uptake of ^{14}C -oleate and BODIPY-FA with a K_m of 200 nM for oleate. Butyrate and 2-Deoxy-glucose uptake in these cell lines were unaltered. Schaffer et al. also showed that FATP was an integral membrane protein and co-fractionated with plasma membrane proteins in 3T3-L1 adipocytes.

Immunofluorescence of 3T3-L1 adipocytes with a FATP antibody showed specific, punctate staining of the plasma membrane. However, a primary criticism of the role of FATP as a fatty acid transporter was its lack of expression in liver and small intestine. This was addressed by Hirsch et al. who showed that FATP was the founding member of a large gene family (Hirsch et al., 1998). They identified six mammalian FATPs (FATP1, FATP2, FATP3, etc...). Homologous genes were also found in many other organisms as well. The function of the FATP gene family was also evolutionarily conserved as expression of the *C. elegans* FATP in *Cos* cells and the *M. tuberculosis* FATP in *E. Coli* increased fatty acid uptake. Importantly, FATP2 and FATP5 were expressed at high levels in the liver and FATP4 was expressed in the small intestine (Hirsch et al., 1998; Stahl et al., 1999). Similar to the data for CD36, FATP4 expression in the small intestine was confined to the enterocytes covering the villi (Stahl et al., 1999). The FATP4 protein was confined to the apical surface of the enterocytes and immuno-electron microscopy showed that it was present in microvilli. Importantly, addition of FATP4 anti-sense oligos to cultured small intestine enterocytes reduced both FATP4 expression and fatty acid uptake suggesting a physiologic role for FATP4 in dietary fatty acid absorption.

However, several groups have suggested that FATPs are very long chain acyl-CoA synthases. Based on peptide sequence from a peroxisomal acyl-CoA synthase purified from peroxisomes, Uchiyama et al. cloned VLACS (very long chain acyl-CoA synthase, also identified as FATP2 by Hirsch et al.) (Uchida et al., 1996; Uchiyama et al., 1996). Although VLACS was 40% identical to Schaffer's FATP and had no significant sequence homology with other acyl-CoA synthases, over-expression of VLACS in Cos cells increased lignoceryl-CoA synthase activity 50-fold. Uchiyama suggested that VLACS had a preference for very long chain fatty acids as palmitoyl-CoA synthase activity was only increased 3-fold. However, the results of this experiment were confounded by the 40-fold higher background of endogenous acyl-CoA synthase activity for palmitate than lignocerate. When expressed in absolute amounts instead of relative fold increase, VLACS had the same activity towards palmitate as lignocerate. Recently, VLACS has also been shown to increase CoA synthase activity for branched chain fatty acids when transiently transfected in Cos cells (Steinberg et al., 1999).

Studies of Fat1p, the yeast FATP homolog, have been similarly controversial. While Faergerman et al. reported that knocking out Fat1p reduced fatty acid uptake by approximately 60%, Watkins et al. showed that VLACS activity in different Fat1p knockout was reduced by 75% (Faergeman et al., 1997; Watkins et al., 1998). Although some data support the hypothesis that Fat1p is a very long chain acyl-CoA synthase, other data in the study by Watkins et al. suggest otherwise (Watkins et al., 1998). First, VLACS activity in yeast may not be due to Fat1p. Although Watkins et al. found equal amounts of VLACS activity in supernatants and organelle pellets, Fat1p is almost certainly an integral membrane protein. Thus, soluble very long chain acyl-CoA synthase activity in the absence of detergent is not due to Fat1p. Second, both Faergerman et al.

and Watkins et al. reported that growth of Fat1p knockout yeast under conditions requiring oleate uptake from the media is impaired. Theoretically, this phenotype could be due to defects in transport, activation, or metabolism of oleate. Although Watkins et al. did not measure oleate CoA synthase activity, palmitate CoA synthase activity was unaffected in the Fat1p knockout. Since the same genes mediate activation of oleate and palmitate, oleate esterification to CoA was probably normal. Moreover, Watkins et al. found that Fat1p yeast did not accumulate long chain fatty acids which also suggests that activation and metabolism of long chain fatty acids was unaffected in the knockout. Thus, the inability of Fat1p knockout yeast to grow on exogenous oleate is probably due to a defect in fatty acid uptake.

The subcellular distribution of the FATP gene family has also become a matter of some dispute. The localization of FATP is significant, as one would expect a fatty acid transporter to be expressed on the plasma membrane. While immunofluorescence and subcellular fractionation showed FATP to be an integral plasma membrane protein, others have suggested that VLACS (FATP2) localizes to peroxisomes in either HepG2 cells or transiently transfected Cos cells (Schaffer and Lodish, 1994; Steinberg et al., 1999). However, immunofluorescence of liver using a VLACS (FATP2) antibody revealed it to be almost exclusively a plasma membrane protein (Stahl et al. unpublished data). Although further data will be needed to definitively address this issue, current data suggest that FATP and VLACS are plasma membrane proteins.

The main issue surrounding the FATP gene family is whether these genes are fatty acid transporters, very long chain CoA synthases, or both. Transfection of VLACS (FATP2) into Cos cells increases fatty acid uptake and very long chain acyl-CoA synthase activity (Hirsch et al., 1998; Uchiyama et al., 1996). Similarly, one yeast knockout suggests that

the yeast FATP homolog is a fatty acid transporter while another study provides data suggesting that Fat1p is a very long chain acyl-CoA synthase. Whether these two activities are performed by the same protein, a complex of associated proteins, or an artifact of over-expression in cell lines will require additional studies.

Adipose Differentiation Related Protein (ADRP)

Adipose differentiation related protein (ADRP) was originally identified as an early marker of adipocyte differentiation (Jiang and Serrero, 1992). The cloned 1.7 kb cDNA predicted a protein of 50kDa had no homology with other proteins. Antibodies to ADRP showed that it was associated with the plasma membrane and the surface of lipid droplets in mature adipocytes. Recently, Gao et al. found that ADRP over-expression in Cos cells increased ^3H -oleate uptake 2.5-fold (Gao and Serrero, 1999). ADRP-stimulated ^3H -oleate uptake had a K_m of 51 nM and, when the uptake assay was performed at 4°C instead of 37°C, ^3H -oleate uptake was reduced by 90%. Analogous to the results of Schaffer et al. for FATP, ADRP increased oleate, palmitate, and arachidonate uptake, but had no effect on either octanoate or 2-deoxy-glucose transport. Although ADRP localized to the plasma membrane in transiently transfected Cos cells and ADRP co-precipitated with the membrane fraction after sub-cellular fractionation, Gao et al. did not provide evidence that ADRP was an integral plasma membrane protein. Whether ADRP directly transports fatty acids across the plasma membrane or increases fatty acid uptake by facilitating intracellular metabolism will be a key question to address in future studies.

Other fatty acid transporters

Two other fatty acid transporters have been reported. In 1987, Fujii et al. identified FAR, FA receptor (Fujii et al., 1987). FAR was purified from rat heart and shown to bind fatty acids *in vitro*. Since the identity of FAR is still unknown, its exact role in fatty acid transport has yet to be determined. Another candidate fatty acid transporter was identified by Trigatti et al. (Trigatti et al., 1991). They incubated adipocyte plasma membranes with a photo-activatable fatty acid analog and found specific labeling of a 22 kDa band. They subsequently identified this protein as caveolin (Trigatti et al., 1999). Caveolin is a highly abundant protein in adipocytes and has been implicated in a variety of cellular processes (Okamoto et al., 1998). Its relevance to fatty acid transport is unclear.

Concluding remarks^c

Do plasma membrane fatty acid transporters exist? Volumes of data suggest that the answer to this question is unequivocally “yes.” cDNAs for FABPpm, CD36, FATP, and ADRP all stimulate fatty acid transport when over-expressed in cells. In each case, the increased fatty acid uptake is saturable and specific for long chain fatty acids. Even though only CD36 and FATP have been shown to be integral plasma membrane proteins, FABPpm and ADRP are clearly associated with the plasma membrane. Thus, almost by definition, all of these proteins are plasma membrane fatty acid transporters. Perhaps the better question is whether these proteins are required for fatty acid transport *in vivo*? The

^c No review of the literature is ever complete or unbiased. To supplement this work, the reader is referred to several recent, excellent reviews (Abumrad et al., 1999; Berk et al., 1996; Berk and Stump, 1999; Fitcher et al., 1996; Glatz et al., 1997; Schaffer and Lodish, 1995).

answer to this question is less clear. Although FABPpm blocking antibodies, SSO cross-linking of CD36, and FATP4 anti-sense oligonucleotides all inhibit fatty acid uptake in primary cultured cells, these experiments are essentially *in vitro*. CD36 knockout mice and Type I CD36 deficiency in humans suggest an important role for CD36 in lipid metabolism *in vivo*. However, as discussed previously, significant issues remain unresolved. Additional study of the CD36 knockout and the creation of knockout mice for the other genes will hopefully shed light on the *in vivo* function of these proteins.

Perhaps an instructive parallel for fatty acid transport can be drawn from carbohydrate metabolism. In the case of glucose transport, two distinct protein families are important. SGLT-1 and 2 are required for glucose uptake in the small intestine and kidney, and Glut1 and Glut4 mediate glucose flux in muscle and fat. While SGLT-1 and 2 are uni-directional transporters which absorb glucose against a concentration gradient, Glut 1 and 4 facilitate the bi-directional diffusion of glucose either into or out of the cell. Similarly, although CD36, FABPpm, FATP, and ADRP all have the same *in vitro* activity, they might have very different physiologic functions. Furthermore, these proteins might act in combination as part of a multi-step process in fatty acid transport.

Fatty acid transport is undoubtedly a complicated process involving many proteins. In addition to plasma membrane transporters, acyl-CoA synthases and FABPs are probably essential for *in vivo* levels of fatty acid uptake. Elucidating the molecular mechanisms for fatty acid transport is likely to provide insight into diseases such as diabetes and obesity. Perhaps a drug targeting one of the genes described above will lead to a better treatment for these diseases.

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Chapter 2

Hormonal regulation of fatty acid uptake by adipocytes

Abstract

Serum fatty acid (FA) levels are dynamically regulated by hormones. Insulin, which rises after a meal, leads to a reduction of serum FAs, while glucagon, which rises in-between meals, stimulates FA efflux from adipocyte stores. Glucagon stimulates FA efflux by activating hormone sensitive lipase in adipocytes. However, insulin's effect on FA flux is less understood. We show here, that within minutes of addition insulin can directly stimulate FA uptake in 3T3-L1 adipocytes. This effect is attenuated by PI-3 kinase and MAP kinase inhibitors, while 24 hour treatment with englitazone, an antidiabetic thiazolidinedione, mimics the effects of insulin. Both TNF- α and FA levels are elevated in obesity and type-2 diabetes, while loss of TNF- α expression increases insulin sensitivity and decreases FA levels. Here we show that TNF- α inhibits basal and insulin-stimulated FA uptake by 3T3-L1 adipocytes. Short-term treatment with TNF- α reduces adipocyte FA uptake by 50% and 24 hour treatment with TNF- α inhibits FA uptake by more than 80%. In conclusion, our data suggest that the insulin-induced drop in serum FA levels is, at least in part, mediated by an increase in FA uptake by adipocytes and that TNF- α can interfere with this process leading to chronically elevated FA levels.

Introduction

Under normal conditions more than 95% of total body triglyceride is sequestered in adipocytes which are approximately 80% triglyceride by weight (Coffee, 1998). Most triglyceride in adipocytes is formed by importing circulating FAs and esterifying them to glycerol. Although the molecular details are unclear, studies of [^3H] oleate uptake by adipocytes demonstrates that free FA uptake is saturable, selective and exhibits many other kinetic properties indicative of facilitated transport (Abumrad et al., 1981). The K_m 's for stearate and oleate uptake are 0.16 and 0.06 μM , respectively, and both FAs have similar V_{\max} values (Abumrad et al., 1984). The K_m for ^{14}C -stearate uptake is the same as the stearate K_i for inhibition of ^3H -oleate uptake (and vice versa for oleate inhibition of [^{14}C]-stearate uptake) suggesting that stearate and oleate uptake proceed through a common protein-mediated pathway. In contrast, [^{14}C] octanoate uptake is 20 times lower than that for oleate and is not competitive by either octanoate or oleate, nor was octanoate able to inhibit uptake of radiolabeled oleate uptake. Also, treatment of membranes with proteases or protein modifying reagents dramatically reduces the facilitated and saturable transport of long chain FAs (LCFAs) into cells, suggesting involvement of one or more proteins (Abumrad et al., 1984; Fraser et al., 1997; Schwieterman et al., 1988).

FA uptake by adipocytes is also altered in rodent models of diabetes and obesity. Uptake of [^3H] oleate in adipocytes from *fafa* rats was 9 times that of normal rats and in adipocytes from the Zucker fatty rats (ZDF), uptake was 13 times that of normal (Berk et al., 1997). Interestingly, these increases were tissue specific as FA uptake in isolated

hepatocytes and cardiomyocytes from either *fa/fa* or ZDF rats was not higher than that of normal rats. Changes in FA uptake can also be observed *in vitro* as differentiation of 3T3-L1 fibroblasts into adipocytes is accompanied by an increase the ability to take up FAs (Schaffer and Lodish, 1994).

Several proteins have been suggested to mediate fatty acid uptake by adipocytes. These include intracellular molecules such as fatty acid binding proteins (FABP), fatty acyl-CoA synthases (FACS), and adipose differentiation related protein (ADRP) . Indeed, over-expression of FACS or ADRP in Cos cells increases fatty acid uptake (Gao and Serrero, 1999; Schaffer and Lodish, 1994). By lowering the intracellular concentration of free fatty acids, these proteins facilitate the movement of fatty acids into the cell. In addition to intracellular molecules, no less than five candidate plasma membrane fatty acid transporters have been proposed (reviewed in (Abumrad et al., 1999; Berk and Stump, 1999; Fitscher et al., 1996)). Three of these, FABPpm, FAT/CD36, and FATP, have been cloned. All increase fatty acid uptake when over-expressed in cell lines and FAT/CD36 and FATP are strongly induced when fibroblast cell lines are differentiated into adipocytes (Abumrad et al., 1993; Ibrahimi et al., 1996; Isola et al., 1995; Schaffer and Lodish, 1994). Adipocytes from FAT/CD36 knockout mice have a reduced ability to take up fatty acids at low fatty acid to albumin ratios (Febbraio et al., 1999). Recently we have shown that FATP is part of a large evolutionarily conserved gene family (Hirsch et al., 1998). Mammals have six homologues, designated FATP1-6, and highly homologous genes are also found in other organisms including flies, worms, fish, and yeast. FATP2 is expressed exclusively in liver and kidney, and FATP5 is expressed only in adult liver. FATP4 is expressed predominantly in the small intestine; it is localized specifically to the brush border of the absorptive epithelial cells and is essential for uptake of LCFAs by

these cells (Stahl et al., 1999). Similar to glucose transport, modulation of one or some of these proteins may alter fatty uptake in adipocytes.

The major metabolic hormones have significant effects on lipid metabolism in adipocytes. Epinephrine and glucagon stimulate the release of FAs from adipocytes by activating hormone sensitive lipase. Insulin suppresses lipolysis and favors FA storage. TNF- α antagonizes the effects of insulin and animals with elevated levels of TNF- α have increased circulating FAs. Here we show that insulin and TNF- α directly regulate FA uptake in 3T3-L1 adipocytes. Insulin stimulation of FA uptake requires PI-3 kinase and TNF- α inhibition of FA uptake can be mimicked by sphingomyelinase. These findings may have implications for the underlying metabolic abnormalities in type II diabetes and obesity.

Material and Methods

Materials- BODIPY-FA (C1-BODIPY-C12) was obtained from Molecular Probes, tritiated palmitate was purchased from NEN, and all other materials were obtained from Sigma.

Cell Culture - 3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were propagated and differentiated to adipocytes as described (Frost and Lane, 1985).

Fluorescent FA uptake assay -FA uptake was assayed with a BODIPY labeled FA analog (BODIPY-FA) Briefly, cells were washed with PBS containing 0.1% BSA (PBS/BSA) 3 times for 5 min followed by an incubation for 2 min at 37 °C in FA uptake solution,

which contained 1 μ M BODIPY-FA in PBS/BSA (13). After two minutes, the cells were washed 4 times with ice cold PBS /BSA. The cells were then removed from the plates with PBS containing 5 mM EDTA and resuspended in PBS containing 10% FCS and 10 mM EDTA. BODIPY-FA fluorescence was measured using a FACScan (Becton Dickinson). Cells were gated on forward scatter (FSC) and side scatter (SSC) to eliminate debris and aggregates from the data. Within this population of cells, adipocytes were identified as a discrete population of cells having a higher side scatter (SSC) than the undifferentiated fibroblasts. For assays measuring the effects of hormones on BODIPY-FA uptake, the data reflects FA uptake by adipocytes.

Efflux Essays- Day 8 3T3-L1 adipocytes were trypsinized and seeded into 48well plates. Cells were incubated overnight with tissue culture medium containing 0.1% BSA and 100 μ M [3 H]-palmitate. Un-incorporated FAs were removed by three washes with PBS/0.1% BSA followed by a 30 minute incubation of the cells with tissue culture medium containing 0.1% BSA and the indicated hormones at 37 °C. Supernatants and cells were removed from the plate and subjected to β -scintillation counting. In order to normalize for well-to-well differences in adipocyte number, efflux was determined for each well by expressing the ratios of activity in the supernatant to the total activity found in the supernatant and the cells. All measurements were done in quadruplicates.

[3 H]-palmitate uptake assays- 3T3-L1 adipocytes were transferred to 6 well plates and allowed to attach overnight. Cells were incubated with the indicated hormones for 15 minutes, washed with PBS/0.1% BSA, and incubated with 50 μ M [3 H]-palmitate in PBS/BSA for 3 minutes at 37 °C. The assay was stopped by transferring the cells to 0°C and extensive washing with ice-cold PBS/BSA. Cells were lysed in 1% SDS and subjected to β -scintillation counting.

Fluorescent Microscopy- 3T3-L1 adipocytes were transferred onto gelatin coated glass coverslips and allowed to adhere overnight. Cells were incubated with BODIPY-FA as described for the FACS assay and visualized using a Zeiss confocal fluorescent microscope.

Results

Heterogeneity of adipocytes for LCFA uptake

BODIPY-FA is a fluorescent LCFA analogue that has been well established as a tool for measuring FA uptake in a variety of cell types. We used 2 minute incubations with BODIPY-FA and fluorescent activated cell scanning (FACS) to quantify initial rates of FA uptake of 3T3-L1 adipocytes and found ((Schaffer and Lodish, 1994) and unpublished data) that incorporation was linear over this period. Previous work showed that differentiation of 3T3-L1 fibroblasts into adipocytes leads to a dramatic increase in rates of LCFA uptake (Abumrad et al., 1991) .While less than 1% of undifferentiated fibroblasts took up significant amounts of BODIPY-FA (data not shown), 50% of 3T3-L1 adipocytes, 10 days after induction of differentiation, showed robust LCFA uptake , i.e. more than 20 arbitrary FL1 units (Fig. 1A). BODIPY-FA uptake by these cells was completely competable with a 100 fold excess of palmitate, indicating a transporter mediated process. Since not all of the fibroblast-like 3T3-L1 cells can be differentiated, we asked whether all of the BODIPY-FA-negative cells are undifferentiated cells. Interestingly, immunofluorescence microscopy showed not only that undifferentiated fibroblasts did not take up LCFAs, but also that a significant proportion of adipocytes, marked with arrows in Fig. 1B and C, also failed to accumulate BODIPY-FA. However,

these cells were morphologically indistinguishable from fatty-acid-transport-competent adipocytes (Fig. 1B and C).

We quantified this heterogeneity by taking advantage of the fact that lipid accumulation in differentiating cells increases their side scatter, one of the parameters measured by the FACS. Fig. 1D shows a typical population of 3T3-L1 cells 10 days after differentiation. Based on differences in side scatter, two groups of cells are apparent. 30% of the cells fall into a low side scatter group corresponding to undifferentiated fibroblasts, while 70% of the cells have a high side scatter indicating lipid accumulation and adipocyte differentiation. Although 70% of the cells have an adipocyte like morphology, only 46% of all cells take up FAs above the baseline value (Fig 1E), indicating that about one quarter of all adipocytes fails to accumulate BODIPY-FA. However, while not every high side scatter cell took up BODIPY-FA, as expected all of the cells which did take up BODIPY-FA were in the high side scatter population (Fig. 1F). Taken together, the results from Fig. 1B, C, and F demonstrate that adipocyte differentiation is required but not sufficient for FA transport.

Insulin and TNF- α control LCFA uptake by adipocytes

To determine whether the number of FA transport competent adipocytes and/or the extent of LCFA uptake by these cells could be regulated, we stimulated 3T3-L1 adipocytes with two hormones, insulin and TNF- α , that have been previously associated with alterations in FA metabolism (Stephens et al., 1997). To this end, 3T3-L1 adipocytes were incubated for 30 minutes with either 50 ng/ml TNF- α or insulin. Subsequently, the cells were rinsed and incubated with albumin bound [3 H]-palmitate for 2 minutes, during which uptake of [3 H] radioactivity was linear (not shown). Insulin treatment of 3T3-L1

cells increased rate of FA uptake nearly two fold, while incubation with TNF- α significantly inhibited LCFA uptake (Fig. 2A).

To study this novel effect of insulin and TNF- α on FA uptake in greater detail, we repeated the experiment using BODIPY-FA instead of [3 H]-palmitate. After 6 hours of serum starvation, LCFA uptake by control cells was significantly lower than cells kept in the presence of serum; 46% of cells maintained in serum showed BODIPY-FA uptake above an arbitrary cut- off of 20 FL1 units , versus 31% of serum starved cells (Fig.2 B I). Short term treatment of serum-starved adipocytes with TNF- α led to a drastic reduction in LCFA uptake. Compared to untreated cells, there was a 50% reduction in the number of cells above the 20 FL1 unit cutoff used to define BODIPY-FA-positive cells (Fig. 2B II). Conversely, insulin not only increased the number of adipocytes deemed BODIPY- positive by 1.6 to 2 fold, but also enhanced the average fluorescence of the positive cells, indicating that the rate of FA uptake in BODIPY-FA positive cells was increased (Fig. 2B III). Short-term treatment of adipocytes with varying concentrations of insulin and TNF- α showed that TNF- α was effective at 50 ng/ml while a significant increase in FA uptake was apparent using insulin concentrations as low as 0.05 ng/ml (Fig. 3A). Interestingly, insulin and, to a greater extent, TNF- α showed a biphasic time course as the short term effects of these hormones are magnified after 8 to 24 hours of incubation (Fig. 3B).

To further characterize the signaling pathways through which either insulin or TNF- α affect LCFA uptake, we measured BODIPY-FA uptake by serum starved 3T3-L1 adipocytes, as shown in Fig. 2B. Treatment with either inhibitors of PI3- or MAP-kinases for 30 minutes significantly reduced the short-term stimulatory effect of insulin on LCFA uptake (Table 1). The antidiabetic thiazolidinedione, englitazone, had no effect

after 10 minutes preincubation but showed an enhancement of LCFA uptake, comparable to that of insulin, after 16 hours of pre-incubation.

Overnight pre-incubation of adipocytes with TNF- α led to a 75% reduction in the number of BODIPY-FA positive cells, compared to insulin stimulated cells. Most cells, including adipocytes [Sethi, 1999 #230], express two TNF- α receptors, a 55 and a 75 kD protein. While murine TNF- α can bind to both receptors on mouse cells, human TNF- α binds only the 55 kD murine receptor. Since human and murine TNF- α inhibited LCFA uptake to a comparable extent (Table 1), the 55 kD TNF α receptor is sufficient for signal transduction. In accordance with this hypothesis, adding either sphingomyelinase or ceramide, known second messengers of the 55 kD TNF- α receptor [Mathias, 1998 #240], mimicked the inhibitory effect of TNF- α (Table 1).

Short-term incubation with insulin or TNF- α do not affect efflux of LCFA from adipocytes

Glucagon stimulates the generation of unesterified intracellular LCFAs from intracellular lipid stores through activation of hormone sensitive lipase *via* phosphorylation by Protein Kinase A following a rise in cAMP. Given that LCFA influx and efflux may use, at least in part, the same cellular transport machinery, stimulation of FA efflux from endogenous lipid stores would effectively reduce the uptake of extracellular BODIPY-FA. Thus, agents which activate hormone sensitive lipase could inhibit BODIPY-FA uptake by stimulating FA efflux. To test whether TNF- α inhibits influx of BODIPY-FA by stimulating lipolysis, we developed a FA efflux assays using 3T3-L1 adipocytes. Efflux of FAs from adipocytes incubated overnight with tritiated palmitate was linear over a 1 hour period (data not shown) and could be inhibited by the addition of unlabeled

palmitate to the medium (Fig 4B). While known stimulators of hormone sensitive lipase, such as isoproterenol and forskolin, caused a marked 2 to 3 fold increase in the efflux of LCFA, neither insulin nor TNF- α induced any change in FA efflux in these assays (Fig. 4 A).

Discussion

Adipocytes are the principal storage site for triglycerides in the body and possess a large capacity to extract FAs from the bloodstream (Coffee, 1998). 3T3-L1 cells are a well-accepted model for fat cell biology (Cowherd et al., 1999). However, we noted a considerable variation in FA uptake among differentiated 3T3-L1 adipocytes. Using a fluorescent FA analogue, BODIPY-FA, we were able to clearly identify two populations of adipocytes; one competent to transport LCFA and another unable to transport them at a significant rate. The reason for this heterogeneity is unknown but cannot be explained by differences in passive uptake of LCFA since we confirmed by fluorescence and phase-contrast microscopy that BODIPY-FA - positive and - negative cells were similar in cell size and number of lipid droplets. Rather, it is likely that differences in the expression of proteins implicated in active uptake of LCFA by adipocytes, such as FATPs, CD36 and LACSs (reviewed in (Abumrad et al., 1999; Berk and Stump, 1999; Fitscher et al., 1996)) are responsible for the heterogeneous uptake of FAs. We clearly showed, using BODIPY-FA in conjunction with flow cytometry sorting, that fully-differentiated, LCFA uptake competent, adipocytes can be easily separated by FACS gating from other cells and used for subsequent experiments.

Insulin levels rise after a meal and counteract the elevation in blood glucose levels by increasing glucose uptake by skeletal muscle and adipose tissue while simultaneously suppressing gluconeogenesis in the liver (Frayn, 1998). Similarly plasma levels of non-esterified FAs quickly fall in response to elevated insulin levels (Frayn, 1998). The mechanisms of this response are currently unknown; however, the data we presented here show that insulin stimulates LCFA uptake of 3T3-L1 adipocytes two- fold within a few minutes. Interestingly, TNF- α , a known desensitizer of insulin signaling (Hotamisligil and Spiegelman, 1994), robustly inhibits FA uptake. Although the ability of chronic TNF treatment to reduce FATP and CD36 expression in adipocytes has been noted before (Memon et al., 1998), to our knowledge, this is the first example of an acute effect on LCFA uptake by a hormone or cytokine.

The mechanisms underlying regulated LCFA uptake into adipocytes are unknown. However based on the time course of insulin and TNF- α action we speculate that the short- term effects are mediated by a direct effect on FA transporter activity or localization on the plasma membrane. We do not know whether FATP1 or any of the other proteins implicated in fatty acid transport is affected by either insulin or TNF- α . Long term effects can be mediated by transcriptional regulation of a FA transporter and/or by controlling the stability of a transport protein. Short-term regulation of LCFA uptake is likely achieved by translocation or covalent modification of FA transporters as neither insulin nor TNF- α changed LCFA efflux from adipocytes. Conversely, glucagon and other known stimulators of hormone sensitive lipase (Holm et al., 1997) robustly stimulated efflux. Since increased efflux blocked LCFA influx at low extracellular palmitate concentrations and since high extracellular palmitate concentrations inhibited LCFA efflux, it is likely that both LCFA influx and efflux share at least one common component which could be a transmembrane FA transporter such as FATP1 (Hirsch et

al., 1998; Schaffer, 1996) or intracellular fatty acid binding proteins such as adipocyte-FABP (Gericke et al., 1997).

Increased TNF- α production by adipose tissue is often associated with obesity and elevated FFA serum levels. We demonstrated that pre-incubation of adipocytes with TNF- α not only inhibited basal LCFA uptake but also completely abolished the ability of insulin to enhance FA uptake by fat cells. This suggests a direct link between increased TNF- α levels and the dysregulation of serum FFAs observed in obesity.

Figure legends

Figure 1 BODIPY-FA uptake in day 8 3T3-L1 adipocytes is heterogeneous and confined to a subpopulation of differentiated adipocytes.

FL1 represents BODIPY-FA fluorescence and is plotted on a logarithmic scale. Higher values indicate more BODIPY-FA uptake. Counts indicate the number of cells. Cells in the figure were not starved and all data was gated on FSC and SSC to exclude debris and cell aggregates. All experiments were performed at least twice with identical results.

- A. BODIPY-FA uptake in day 8 adipocytes is specific. BODIPY-FA uptake in day 8 adipocytes was measured as detailed in Materials and Methods (blue line). In another plate of cells, 100 μ M palmitate was added to the assay mixture (red line).
- B. BODIPY-FA uptake in day 8 adipocytes is heterogeneous. BODIPY-FA uptake in day 8 adipocytes was performed as described in Materials and Methods. Green fluorescence indicates BODIPY-FA uptake. Nuclei of cells were stained with propidium iodide. Arrows indicate BODIPY-negative cells which can be directly compared to those in panel C. A representative field is shown and this experiment was replicated three times.
- C. Phase contrast image of the same field as part B.
- D. Physical heterogeneity of Day 8 3T3-L1 adipocytes. Day 8 adipocytes were treated with BODIPY as described in Materials and Methods. Shown here is the forward- (FSC) and side-scatter (SSC) plots. The black line represents an arbitrary value distinguishing distinct high SSC and low SSC populations and the percentages indicate the fraction of the total cells that these two populations represent.

- E. BODIPY-FA uptake by the entire cell population. BODIPY-FA fluorescence of cells from panel D is depicted. R1 is an arbitrary fluorescence value which divides the cells into BODIPY-FA positive and BODIPY-FA negative populations. The percentage reflects the fraction of the cells contained within R1. Cells in this figure include both cell populations depicted in part D.
- F. Only differentiated adipocytes take up significant amounts of BODIPY-FA. The FSC and SSC of the R1 population is plotted. The black line is drawn at the same value as in panel D and the percentages represent the fraction of R1 positive cells in the high SSC and low SSC populations respectively.

Figure 2 Hormonal regulation of [^3H]-palmitate uptake by 3T3-L1 adipocytes.

- A. [^3H]-palmitate uptake over the first 3 min. was measured as described in Materials and Methods. Uptake was measured following a 15 minute pretreatment with either 50 ng/ml insulin, or 50 ng/ml TNF- α . Untreated cells were used as a control. The effects of insulin and TNF are normalized to untreated cells which were set to represent 100% control uptake.
- B. 3T3-L1 adipocytes were starved overnight and BODIPY-FA uptake was measured as described in Materials and Methods. FL1 represents BODIPY-FA fluorescence and is plotted on a logarithmic scale. The data shown represents BODIPY-FA uptake only by adipocytes which were identified by having a high SSC value. The region depicted represents an arbitrary line to separate the cells into BODIPY-FA positive and BODIPY-FA negative populations. The same region was used in all three plots and the number corresponds to the percent of the cells in the plot which fall within that region. I represents untreated cells. II, cells were treated with 50 ng/ml TNF- α .

for 15 minutes before BODIPY-FA addition. III, cells were treated with 50 ng/ml insulin for 15 minutes. This experiment was performed three times and a representative experiment is shown.

Figure 3 Hormone effects on LCFA uptake

BODIPY-FA uptake was measured after overnight starvation of 3T3-L1 adipocytes. Cells were gated for adipocytes as described, and an arbitrary value of BODIPY-FA fluorescence was used to split the adipocytes into BODIPY-FA positive and BODIPY-FA negative populations. The percentage of BODIPY-FA positive control cells was set to 100% and uptake by drug treated cells were normalized to the 100% value of control cells. White bars represent insulin treatment and black bars represent TNF- α treatment. This experiment was performed three times with similar results.

- A. Dose response of insulin and TNF- α on FA uptake. The cells were treated with the indicated amounts of insulin or TNF- α for 15 minutes and FA uptake was measured as previously described.
- B. Time course of insulin and TNF- α on FA uptake. Cells were placed in serum free media and incubated with either 50 ng/ml insulin or TNF- α for the indicated lengths of times.

Figure 4 FA efflux from 3T3-L1 adipocytes.

3T3-L1 adipocytes were incubated in the presence of [3 H]-palmitate overnight and FA efflux was measured as described in Materials and Methods.

- A. Regulation of [3 H]-palmitate efflux. Cells were loaded overnight with [3 H]-palmitate as described in the Materials and Methods section and efflux was normalized to that of control cells. The concentrations used were 50 ng/ml insulin, 50 ng/ml TNF- α , 10

μM isoproterenol, 10 μM forskolin. The data shown represent the average of quadruplicates. Bars indicate the standard error of the mean.

- B. FA efflux was determined as before but in the presence of the indicated amounts of extracellular palmitate. BSA concentration was kept constant at 0.1%. The data shown represent the average of quadruplicates. Bars indicate the standard error of the mean.

Table 1 Modulation of LCFA uptake.

Short term FA uptake was determined as described in the legend to Fig. 2B after 3T3-L1 adipocytes were incubated for the indicated amount of time with cytokines and/or inhibitors. Concentrations used were, 50 ng/ml insulin, 500 nM wortmannin, 10 nM PD98057, 50 ng/ml human or murine TNF- α , 30 μM Englitazone, 1 U/ml sphingomyelinase (added to intact cells (Peraldi et al., 1996)), 1 μM hexa-ceramide (a cell permeable ceramide derivative (Peraldi et al., 1996)), 50 ng/ml glucagon, 10 μM forskolin, 10 μM isoproterenol, 50 μM AIF4, 100 μM 8-Br-cAMP

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Fig 1

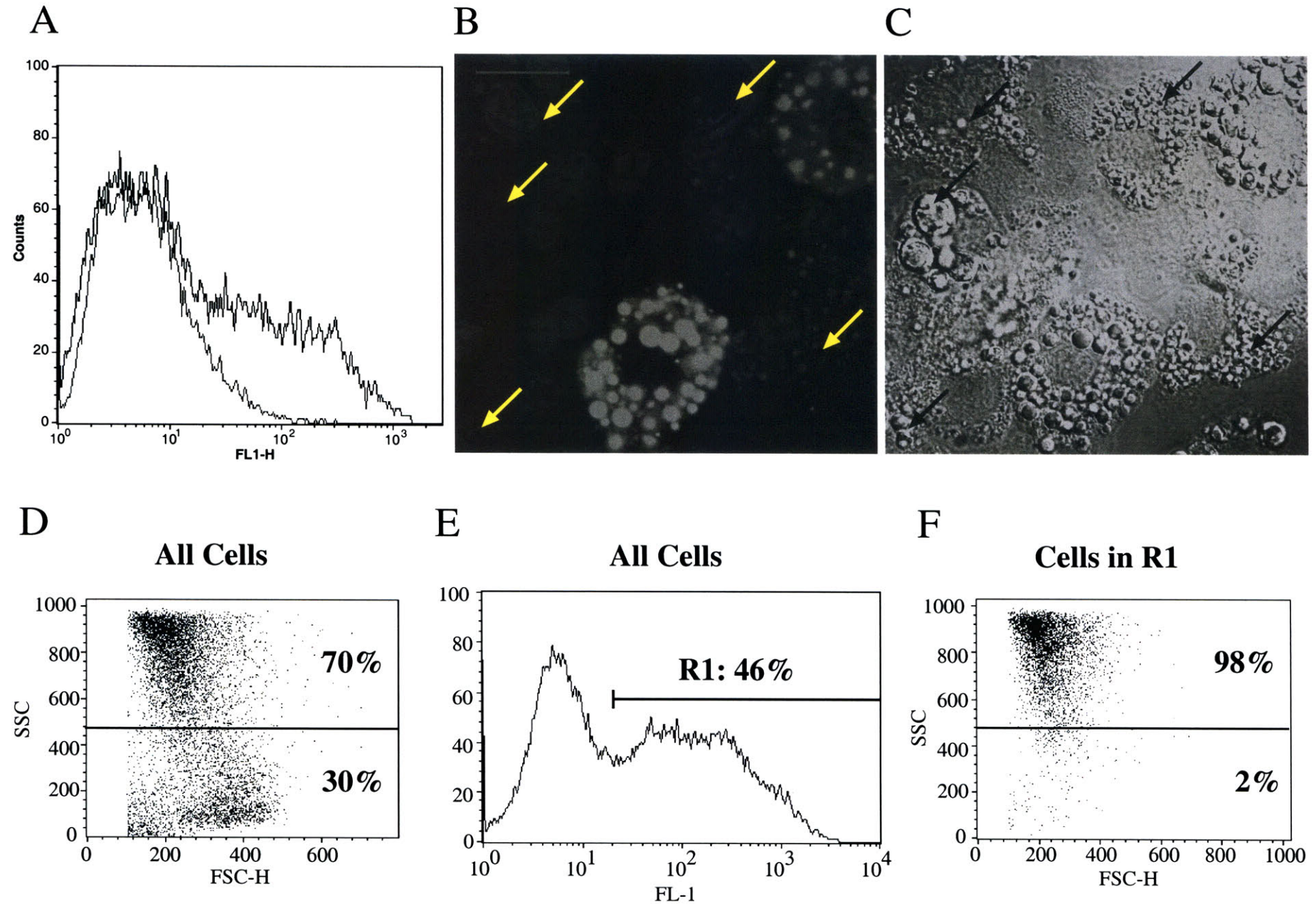
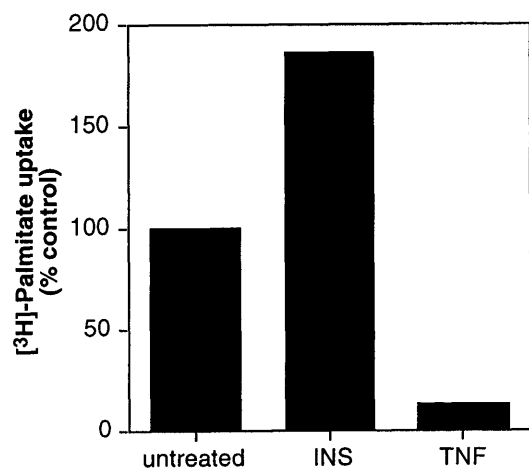


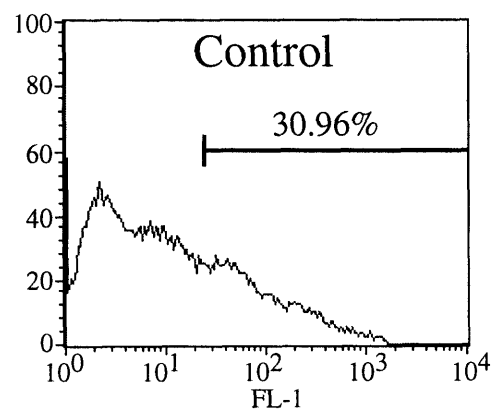
Fig 2

A

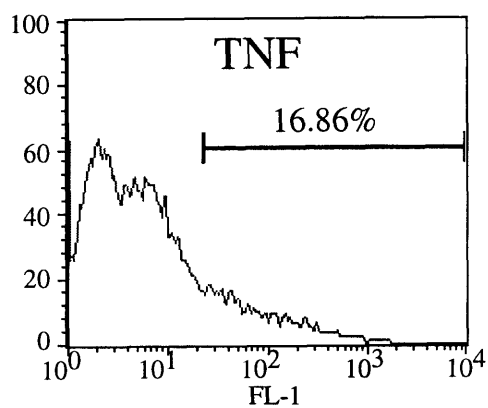


B

I



II



III

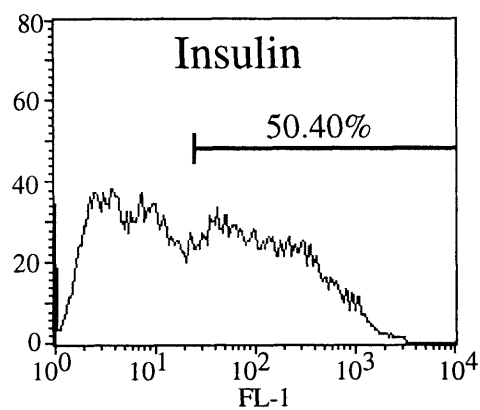
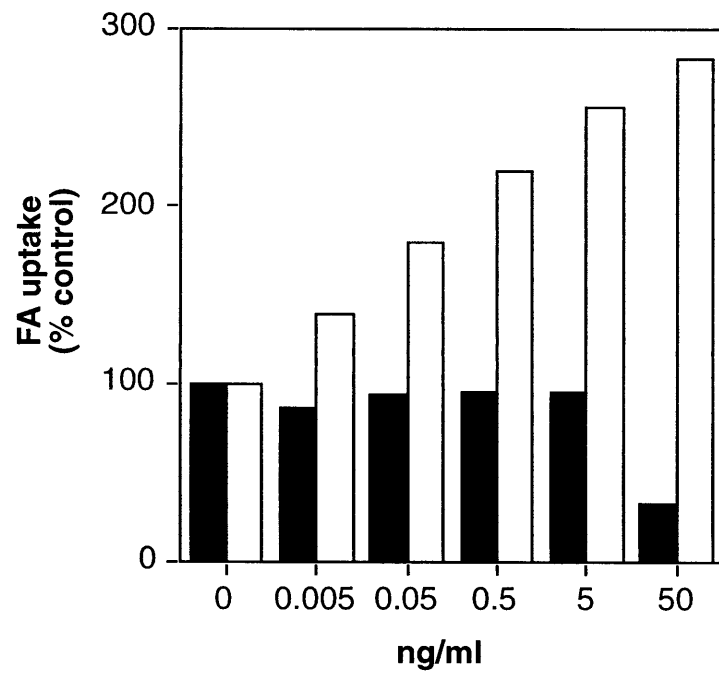


Fig 3

A



B

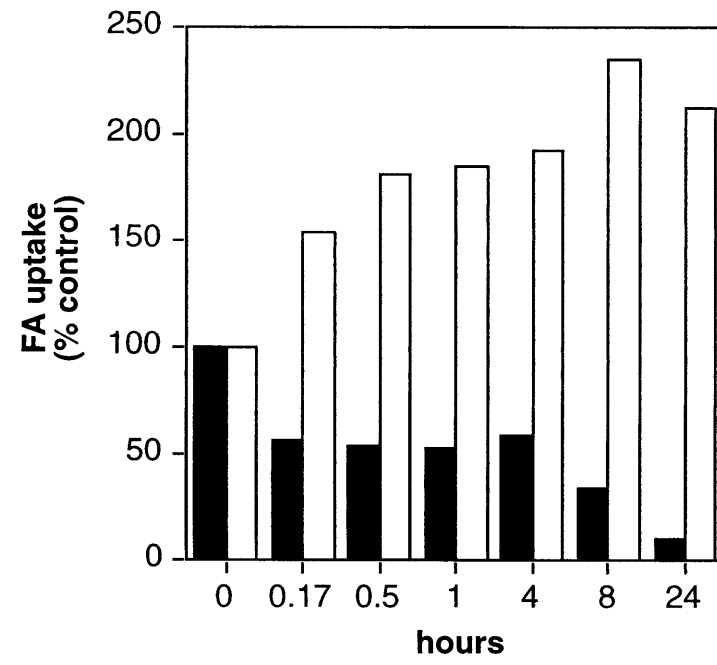
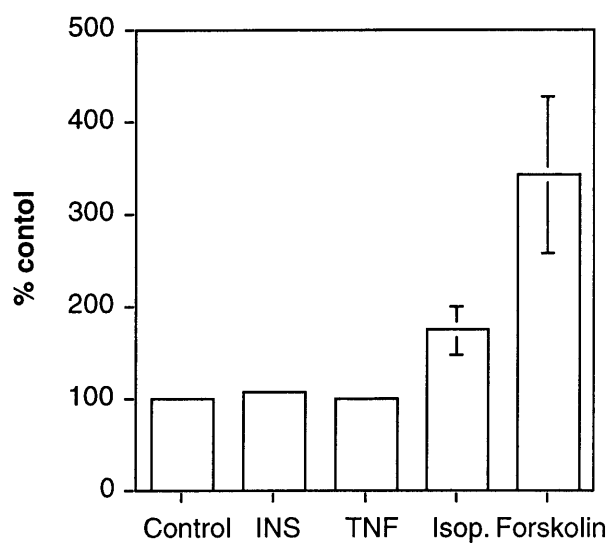


Fig 4

A



B

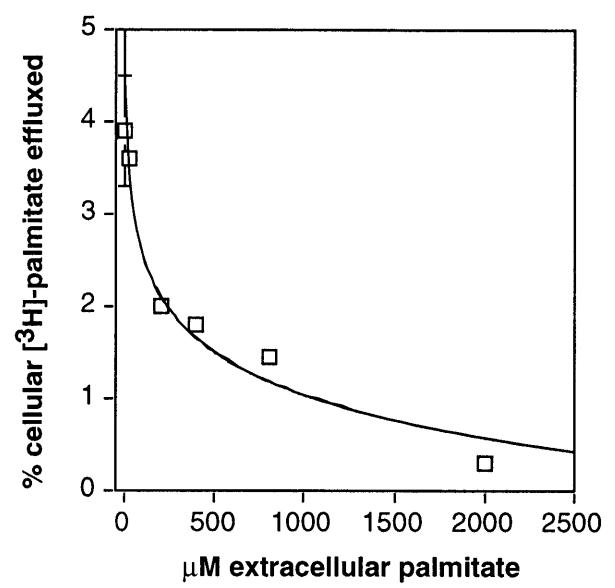


Table 1

Pre-Incubation	Treatment	LCFA uptake (% control)
10 min	insulin	181
30 min + 10 min	wortmanin + insulin	119
30 min + 10 min	PD98057 + insulin	95
16 h + 10 min	TNF- α + insulin	60
10 min	Englitazone	95
16 h	Englitazone	185
10 min	murine TNF- α	41
10 min	human TNF- α	50
10 min	sphingomyelinase	29
10 min	ceramide	32
10 min	glucagon	14
10 min	forskolin	7
10 min	isoproterenol	12
10 min	AlF ₄	25
10 min	8-Br-cAMP	33

Chapter 3

A family of fatty acid transporters conserved from mycobacteria to man

Abstract

Long chain fatty acids (LCFA) are an important source of energy for most organisms. They also function as blood hormones, regulating key metabolic functions such as hepatic glucose production. While LCFAs can diffuse through the hydrophobic core of the plasma membrane into cells, such non-specific transport cannot account for the high affinity and specific transport of LCFAs exhibited by cells such as cardiac muscle, hepatocytes and adipocytes. Transport of LCFA across the plasma membrane is facilitated by FATP, a plasma membrane protein that increases LCFA uptake when expressed in cultured mammalian cells (Schaffer and Lodish, 1994). Here we report the identification of four novel murine FATPs, one of which is expressed exclusively in liver and another only in liver and kidney. Both genes increase fatty acid uptake when expressed in mammalian cells. All five murine FATPs have homologues in humans in addition to a sixth FATP gene. FATPs are found in such diverse organisms as *F. rubripes*, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, and *M. tuberculosis*. The function of the FATP gene family is conserved throughout evolution as the *C. elegans* and mycobacterial FATPs facilitate LCFA uptake when over-expressed in COS cells or *E. coli*, respectively. The identification of this evolutionarily conserved fatty acid transporter family will allow us to gain a better understanding of the mechanisms whereby LCFAs traverse the lipid bilayer as well as yield insight into the control of energy homeostasis and its dysregulation in diseases such as diabetes and obesity.

* Abbreviations: LCFA, Long Chain Fatty Acid; FATP, Fatty Acid Transport Protein; VLACS, Very Long chain Acyl-CoA Synthase.

Introduction

Long chain fatty acids (LCFA) are an important energy source for pro- and eukaryotes and are involved in diverse cellular processes such as membrane synthesis, intracellular signaling, protein modification, and transcriptional regulation. In developed Western countries human dietary lipids are mainly di- and triglycerides and account for approximately 40% of caloric intake (Weisburger, 1997). These lipids are broken down into fatty acids and glycerol by lingual, gastric, and pancreatic lipases (Chapus et al., 1988); in the small intestine, long chain fatty acids are then transported into brush border cells where the majority is re-esterified and secreted into the lymphatic system as chylomicrons (Green and Riley, 1981). Fatty acids are liberated from lipoproteins by the enzyme lipo-protein lipase which is bound to the luminal side of endothelial cells (Scow and Blanchette-Mackie, 1992). “Free” fatty acids in the circulation are bound to serum albumin (Spector, 1984) and are rapidly incorporated by adipocytes, hepatocytes and cardiac muscle cells. The latter derive 60-90% of their energy through the beta oxidation of long chain fatty acids (Neely et al., 1972). Although saturable and specific uptake of LCFA has been demonstrated for intestinal cells, hepatocytes, cardiac myocytes and adipocytes the molecular mechanisms of LCFA transport across the plasma membrane have remained controversial (Hui and Bernlohr, 1997; Schaffer and Lodish, 1995). Five proteins have been suggested to mediate LCFA uptake into cells. Four candidate LCFA transporters, FABPpm (Stremmel et al., 1985), 56 kD renal FABP (Fujii et al., 1987), Caveolin (Trigatti et al., 1991), and FAT (Abumrad et al., 1993), were identified by their ability to bind fatty acids. The fifth, fatty acid transport protein (FATP), was identified by Schaffer and Lodish using an expression cloning strategy (Schaffer and Lodish, 1994). FATP is a 63 kD plasma membrane protein which increases LCFA uptake when stably expressed in cell lines but has no effect on either glucose or short chain fatty acid transport. FATP is induced during adipocyte differentiation *in vitro* and is expressed in brain, skeletal muscle, heart, fat, and kidney but not liver (Schaffer and Lodish, 1994). Since the liver has a large capacity for fatty acid uptake (Stremmel, 1989), we started a search for FATP homologues. Here we describe a large family of highly homologous mammalian LCFA transporters which are widely expressed including all tissues relevant to fatty acid metabolism. Furthermore, we also identified novel members of this family in other species including mycobacterial and nematode FATPs which, like their mammalian counterparts, are functional fatty acid transporters.

Materials and Methods

Sequence alignment of FATP clones. The DNA sequence for FATP1 was obtained from the NCBI non-redundant database. cDNAs for mmFATP2, 3, 4, and 5 were obtained by screening mouse expression libraries, purchased from Gibco/BRL, with probes derived from the cloned ESTs (Research Genetics). Full length clones were obtained for mmFATP2 and 5 and partial sequences for FATP3 and 4. Neither FATP2 or FATP5 contains an in-frame stop codon upstream of the putative initiator methionine; initiator methionines were assigned by homology with that in mmFATP1 and by the presence of a signal sequence immediately after it. The *M. tuberculosis*, *C. elegans* and *S. cerevisiae* sequences were present in the dbEST database as part of the sequencing projects for these organisms. Sequences were aligned utilizing a ClustalX algorithm and the resulting alignment exported to SeqVu. Homologous amino acid substitutions are shaded and were determined using the Dayhoff 250 method with a 50% homology cutoff.

COS cell transfection and LCFA uptake. COS cells were co-transfected using the DEAE-dextran method with the mammalian expression vector pCDNA 3.1 (Invitrogen) expressing the gene for CD2 (pCDNA-CD2) together with either a pCDNA 3.1 or pCMVSPORT2 (Gibco/BRL) expression vector containing one of the murine or nematode FATP genes (pCDNA-mmFATP1, pCDNA-FATP2, pCMVSPORT-FATP5, pCDNA-ceFATPb). Two days after transfection, cells were assayed for CD2 expression with a phycoerythrin coupled anti-CD2(PE-CD2) monoclonal antibody (Pharmingen) and fatty acid uptake was assayed with a BODIPY-labeled fatty acid analog (Molecular Probes). Briefly, cells were washed twice with PBS and stained with PE-CD2 at 4° C for 30 min in PBS containing 10% fetal calf serum (PBS/FCS). They were then washed with PBS/FCS 3 times for 5 min followed by an incubation for 2 min at 37 °C in fatty acid uptake solution, which contained 0.1 µM BODIPY-FA and 0.1% fatty acid free BSA in PBS (Schaffer and Lodish, 1994). After two minutes, the cells were washed 4 times with ice cold PBS/0.1% BSA. The cells were then removed from the plates with PBS containing 5 mM EDTA and resuspended in PBS containing 10% FCS and 10 mM EDTA. PE-CD2 and BODIPY-FA fluorescence were measured using a FACScan (Becton Dickinson). COS cells were gated on forward scatter (FSC) and side scatter (SSC). Cells exhibiting over 300 CD2 fluorescence units representing ~15% of all cells were deemed CD2 positive and their BODIPY-FA fluorescence was quantitated.

E. coli based LCFA uptake assay. The full length coding region of mtFATP and a control protein, the mammalian transcription factor TFE3, were subcloned into the inducible, prokaryotic expression vector pET (Novagen). Expression was induced with 1 mM IPTG for 1 hour or cells

were left uninduced. Cells were washed in PBS/0.1% BSA and resuspended in 1 ml PBS/0.1% BSA containing 0.1 μ M [3 H]palmitate (NEN) at 37°C. Uptake was stopped after the indicated incubation time by transferring the cells onto filter paper using a cell harvester (Brandel). Filters were washed extensively with ice cold PBS/0.1% BSA and [3 H]palmitate was quantitated by scintillation counting

Northern Blots. Northern blot analysis of murine FATP expression was done using poly-A mRNA blots (Clontech). Probes for each of the FATPs were derived from the 3' UTR regions of each gene and were less than 60% identical in sequence. Probes were labeled by random priming (Mannheim Boehringer) and hybridized at 65°C. Blots were extensively washed in 0.2% SSC/0.1% SDS at 65°C.

Generation of Phylogenetic Trees. Complete and partial sequences for FATP genes from human, rat, mouse, puffer fish, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and *M. tuberculosis* were aligned using ClustalX. A homologous region of 48 amino acids (residues 472 to 519 in mmFATP1) from all of the genes was used to determine phylogenetic relationships within ClustalX. Based on these data a phylogenetic tree was generated using TreeViewPPC.

Results

Identification of novel mammalian FATPs.

To identify novel FATPs we screened the NCBI expressed sequence tag (EST) database using the mouse FATP protein sequence. This strategy led to the identification of over 50 murine EST sequences which we could assemble into five distinct contiguous DNA sequences (contigs). One contig was identical to the previously cloned FATP, which we have renamed FATP1. Another, which we have renamed FATP2, is the murine homologue of a rat gene previously identified by others as a very long chain acyl-CoA synthase (Uchiyama et al., 1996). The other three contigs represented novel genes (FATP3, 4, and 5). By screening cDNA libraries made from mouse day 10.5 embryos and adult liver we obtained full length clones for FATP2 and FATP5 and nearly complete sequences for FATP3 and 4 (Fig. 1). We also identified human homologues for each of the murine genes in the EST database. Additionally, we found a sixth human gene; whether this gene is also present in the mouse will require additional studies.

We assessed the ability of the newly identified mouse genes to function as fatty acid transporters using a fluorescence-activate cell scanning (FACS)-based assay. COS cells were

transiently co-transfected with expression vectors encoding the cell surface protein CD2 and either FATP1, FATP2 or FATP5, respectively. Two days after transfection, COS cells were stained with an antibody to CD2 and then incubated with a BODIPY-labeled fatty acid (BODIPY-FA, (Schaffer and Lodish, 1994)). The cells were then washed extensively, lifted off the dish and analyzed by FACS. As judged by the number of CD2- positive cells, our transfection efficiency was approximately 20-30% (Fig. 2A). We quantitated fatty acid uptake in the transiently transfected COS cells by measuring the BODIPY-FA fluorescence of the CD2-positive cells. Expression of CD2 had no effect on fatty acid uptake as COS cells expressing only the transfected CD2 cDNA (CD2- positive) had the same low level of BODIPY-FA uptake as did untransfected (CD2- negative) control cells (Fig. 2A, control). In COS cells cotransfected with CD2 and FATP1, FATP2, or FATP5, uptake of BODIPY-FA by the transfected (CD2-positive) cells was increased between 15 to 90-fold over control (CD2 cDNA only) cells (Figs. 2A and B).

Expression patterns of murine FATPs.

We characterized expression patterns of the murine FATP gene family by Northern blot analysis (Fig. 3); to avoid cross-hybridization, we utilized probes from the 3' untranslated region of these genes which shared no appreciable homologies with each other. The expression pattern of FATP1 agrees with that previously found (Schaffer and Lodish, 1994). FATP2 is expressed exclusively in liver and kidney, which corresponds to the reported tissue distribution of the rat homologue (VLACS) as assessed by Western blotting (Uchiyama et al., 1996). FATP3 is present in lung, liver and testis; FATP4 is expressed in heart, brain, lung, liver, and kidney. FATP5 is expressed only in liver and cannot be detected in other tissues even when the blot is overexposed. The human homologue of FATP5 is also liver specific and is not expressed in a wide array of other tissues tested including fetal liver (data not shown).

FATPs are evolutionarily conserved.

Using sequences conserved among the five murine FATP genes we searched the EST database for FATP genes in other organisms and found two homologues in *C. elegans* and one in *Mycobacterium tuberculosis*. We cloned one of the *C. elegans* genes from a cDNA library and expressed it in COS cells as described for the murine FATPs. Interestingly, over-expression of the nematode FATP resulted in a 15-fold increase of BODIPY-FA uptake compared to control cells (Fig. 2B). We then isolated the mycobacterial FATP gene from a phage library and assessed its ability to facilitate fatty acid uptake. *E. coli* transformed with a prokaryotic, IPTG-inducible expression vector containing the mycobacterial FATP gene demonstrated a significant increase in

the rate of [^3H]palmitate uptake after induction compared to uninduced bacteria or *E. coli* transformed with a control protein (Fig. 2C). Novel FATP genes were also identified in *F. rubripes* (puffer fish) and *D. melanogaster*.

Nomenclature.

We propose that the FATP genes be given a species specific prefix (mm, *Mus musculus*; hs, *Homo sapiens*; mt, *Mycobacterium tuberculosis*; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans*, sc, *Saccharomyces cerevisiae*) and numbered such that mammalian homologues in different species share the same number but differ in their prefix. Since the two *C. elegans* genes cannot be paired with a specific human or mouse FATP, we propose that they be labeled ceFATPa and ceFATPb.

Phylogenetic tree of FATPs.

Faergeman et al. identified three regions of very strong conservation between the scFATP and mmFATP1 genes (Faergeman et al., 1997). We compared the sequences of the FATPs over a 311 amino acid FATP “signature sequence” which includes these conserved regions corresponding to AA 246 to 557 in mmFATP1 (underlined blue in Fig 1). When compared to the NCBI non-redundant database, only one region of the “FATP signature sequence” shows significant homology to other proteins. This small stretch of amino acids (underlined red in Fig. 1) is an AMP-binding motif found in a multitude of other proteins such as acyl CoA synthase, several CoA ligases, and gramicidin S synthetase component II (GS2) (Schaffer and Lodish, 1994). The relevance of this motif to fatty acid transport is unclear. Other highly conserved regions among the FATPs, including long stretches of amino acids over 90% identical from mycobacteria to humans, are not found in any other class of proteins. We utilized a 48 amino acid segment of the FATP “signature sequence” to construct a phylogenetic tree (Fig. 4). Each of the human and mouse genes form their own branch; hsFATP6, which as yet has no murine homologue, is most closely related to hsFATP3 and mmFATP3. As expected, mVLACS is closer in sequence to mmFATP2 than to hsFATP2. The FATP genes of invertebrates, i.e. *C. elegans* and *D. melanogaster*, are most closely related to each other. Surprisingly, the mycobacterial gene is more closely related to the human and mouse FATP5 genes than to the FATPs of any of the lower organisms. Whether this reflects co-evolution of the mycobacterial and human genes awaits further study.

Discussion

Here we showed that FATPs are a large evolutionarily conserved family of proteins that mediate the transport of LCFAs into cells. We identified 6 human and 5 mouse FATP genes which are expressed in a variety of tissues. These include the liver specific gene, FATP5, as well as FATP2, which is highly expressed in liver and kidney. We demonstrated that both of these proteins, as well as FATPs from nematodes and mycobacteria, are functional LCFA transporters. Interestingly, mmFATP2 is the murine homologue of a rat gene previously identified by others as a very long chain acyl-CoA synthase (Uchiyama et al., 1996) even though it shares no sequence homology with other cloned acyl-CoA synthases. VLACS increased very long chain acyl-CoA synthase activity when expressed in COS cells (Uchiyama et al., 1996) but this observation can be explained by our finding that expression of mmFATP2, the mouse homologue of VLACS, increases the rate of fatty acid uptake from the medium; this, in turn, could stimulate transcription of a VLACS gene. Indeed, exogenously added LCFAs directly activate transcription of the long-chain acyl-CoA synthase gene (Suzuki et al., 1990).

Not every organism utilizes an FATP to transport free fatty acids. The *E. coli* genome does not contain an FATP homolog; rather, the *E. coli* fadL gene product mediates uptake of fatty acids from the medium (Black and DiRusso, 1994). This protein has no sequence similarity to FATPs and we cannot find homologues of fadL in the genomes of mycobacteria, yeast, *C. elegans*, mice, or humans.

The discovery of a diverse but highly homologous family of FATPs is reminiscent of the glucose transporter family. In a manner similar to the FATPs, the glucose transporters have very divergent patterns of tissue expression (McGowan et al., 1995). The FATPs, like glucose transporters, may also differ in their substrate specificities, uptake kinetics, and hormonal regulation (Thorens, 1996). Indeed, the levels of fatty acids in the blood, like those of glucose, can be regulated by insulin and are dysregulated in diseases such as non-insulin-dependent diabetes and obesity (Boden, 1997). The underlying mechanisms for the regulation of free fatty acid concentrations in the blood are not understood but could be explained by hormonal modulation of FATPs.

Another finding with potentially broad implications is the identification of a FATP homologue in *M. tuberculosis*. Tuberculosis causes more deaths worldwide than any other infectious agent and drug resistant tuberculosis is re-emerging as a problem in industrialized nations (Bloom and Small, 1998). The *de novo* synthesis of fatty acids in *Mycobacterium leprae* is insufficient to maintain growth (Wheeler et al., 1990) and deletion of the scFATP gene slows their growth under certain conditions. Thus, it may be worthwhile to investigate inhibitors of mtFATP as potential therapies for tuberculosis.

Figure Legends

Figure 1. Sequence alignment of FATP clones.

Figure 2. LCFA uptake assays.

A. COS cells were co-transfected using the DEAE-dextran method with the mammalian expression vectors pCDNA-CD2 either alone (Control) or together with one of the FATP containing expression vectors (pCDNA-mmFATP1, pCDNA-mmFATP2 or pCMV-SPORT2-mmFATP5) as described in Materials and Methods. COS cells were gated on forward scatter (FSC) and side scatter (SSC) and the results shown represent >10,000 cells. Cells exhibiting over 300 CD2 fluorescence units (vertical line) representing ~ 15% of all cells were deemed CD2 positive.

B. As in Panel A, COS cells were co-transfected with pCDNA-CD2 either alone (Control) or together with one of the FATP- containing expression vectors (pCDNA-mmFATP1, pCDNA-mmFATP2, pCMV-SPORT2-mmFATP5 or pCDNA-ceFATPb). The mean BODIPY-FA fluorescence of the CD2-positive cells is plotted; results shown represent the average of 3 experiments each consisting of over 50,000 COS cells. Note that a logarithmic scale is used on the ordinate.

C. The full length coding region of mtFATP (squares) and a control protein (TFE3; circles) were subcloned into the inducible, prokaryotic expression vector pET (Novagen). Expression was induced (solid symbols) with 1 mM IPTG for 1 hour or cells were left uninduced (open symbols). Data points were done in triplicate and counts were normalized to the number of bacteria as determined by OD₆₀₀.

Figure 3. Northern blot analysis of murine FATP expression was analyzed using poly-A mRNA blots (Clontech). Probes for each of the FATPs were derived from the 3' UTR regions of each gene and were less than 60% identical in sequence.

Figure 4. Complete and partial sequences for FATP genes from human, rat, mouse, puffer fish, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and *M. tuberculosis* were aligned using ClustalX. Based on these data a phylogenetic tree was generated using TreeViewPPC. The bar indicates the number of substitutions per residue, *i.e.* 0.1 corresponds to a distance of 10 substitutions per 100 residues.

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Fig 1

[illegible]

Fig 2

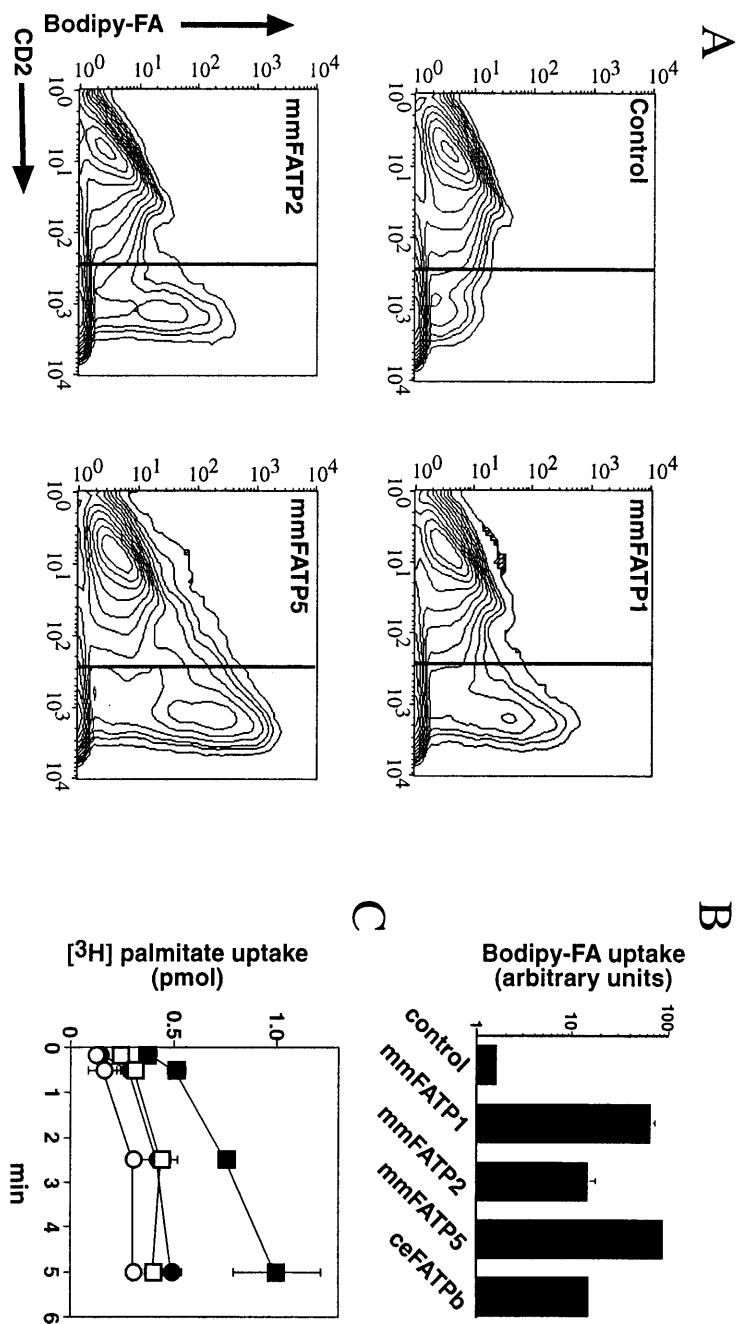


Fig 3

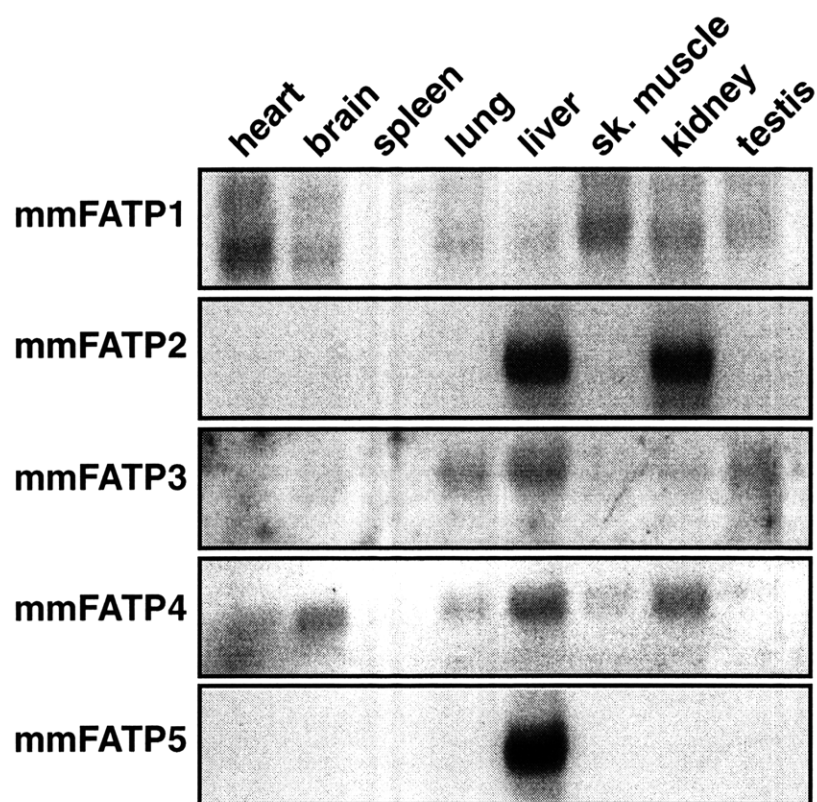
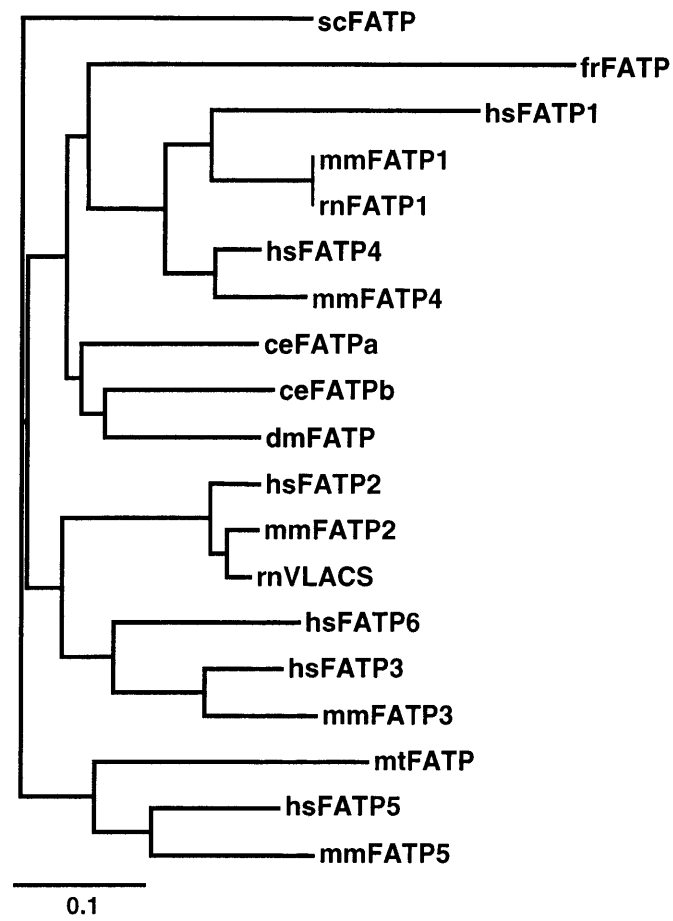


Fig 4



Chapter 4

Identification of the major intestinal fatty acid transport protein

Abstract

While intestinal transport systems for metabolites such as carbohydrates have been well characterized, the molecular mechanisms of fatty acid (FA) transport across the apical plasmalemma of enterocytes have remained largely unclear. Here we show that FATP4, a member of a large family of FA transport proteins (FATPs) is expressed at high levels on the apical side of mature enterocytes in the small intestine. Furthermore, over-expression of FATP4 in 293 cells facilitates uptake of long chain FAs with the same specificity as enterocytes, while reduction of FATP4 expression in primary enterocytes by anti-sense oligonucleotides inhibits FA uptake by 50%. This suggests that FATP4 is the principal fatty acid transporter in enterocytes and may constitute a novel target for anti-obesity therapy.

Introduction

Fats, mainly in the form of di- and triglycerides, contribute over 40% of the caloric content of western diet (Clandinin et al., 1991). Efficient intraluminal digestion and absorption predominantly in the jejunum and also ileum allow less than 5% of the ingested lipids to escape with feces (Carey et al., 1983). Lipases, mainly pancreatic lipase, liberate fatty acids from the lipid droplets in the small intestine (Verger et al., 1996) which then form mixed micelles with bile acids. Long-chain fatty acids (LCFAs) are absorbed by the epithelial cells of the small intestinal villi termed enterocytes (Windler and Greten, 1989), re-esterified and incorporated into chylomicrons as triglyceride. Chylomicrons are formed in the ER of enterocytes and undergo exocytosis at the baso-lateral side of the cell where they subsequently enter the lymphatic system (Tso and Balint, 1986).

The uptake of many nutrients including amino acids, di- and tripeptides, and many vitamins and minerals is mediated by energy-coupled transporters on the apical side of the enterocyte while other molecules, such as

fructose, enter the epithelial cells via facilitated diffusion (Thorens, 1993). Glucose entry into the enterocyte is mediated by SGLT1, a sodium coupled glucose transporter in the brush border membrane of enterocytes (Hediger et al., 1987). Most of the glucose taken up by enterocytes is not modified and exits the cell following a concentration gradient through Glut 2, a facilitative glucose transporter expressed baso-laterally (Thorens, 1992). Subsequent glucose transport in other organs such as liver, muscle, and kidney is then mediated by various members of the Glut family (Thorens, 1996).

Although there are well-characterized examples of transporter families for amphipathic molecules such as bile acids (Shneider, 1998; Suchy et al., 1997), it was initially believed that LCFAs are absorbed by the intestinal epithelial cells through mere diffusion (Green and Riley, 1981; Ling et al., 1989). However, there is now ample evidence that in addition to this diffusion component the intestine (Gore et al., 1994; Stremmel, 1988), liver (Stremmel, 1989), heart (Sorrentino et al., 1988; Stremmel, 1988), adipose tissue (Schaffer and Lodish, 1994) and other organs express a saturable and competent LCFA transport system (Abumrad et al., 1998).

Using an expression cloning strategy our lab had previously identified a membrane protein, fatty acid transport protein (FATP), from murine adipocytes which facilitates the uptake of LCFAs (Schaffer and Lodish, 1994). Subsequently, we reported the discovery of a large family of FATPs characterized by the presence of a FATP signature sequence (Faergeman et al., 1997; Hirsch et al., 1998). Human and mouse FATPs have unique expression patterns and are found in major organs of fatty acid metabolism such as adipose tissue, liver, heart and kidney (Hirsch et al., 1998). So far, five distinct FATPs in mice and six different FATPs in humans have been identified and designated mmFATP1 through 5 and hsFATP1 through 6 respectively (Hirsch et al., 1998). Here we show that a member of this novel family, FATP4, mediates the efficient uptake of fatty acids by enterocytes.

Experimental Procedures

Isolation of hsFATP1 and mmFATP4. Clones encoding full-length human FATP1 and FATP4 were obtained from a heart and a spleen cDNA library respectively by searching Millennium databases for sequences similar to murine FATP1-5 coding regions using the BlastX algorithm (Altschul et al., 1990).

Generation of cell lines stably expressing hsFATP4. A DNA fragment containing the entire hsFATP4 coding sequence as well as 100 nucleotides of the 5' and 50 nucleotides of the 3' untranslated region was inserted into the vector pIRES-neo (Clontech). The resulting construct or a vector control (pIRES-neo) was transfected into 293 cells using the lipofectamine method (Gibco BRL) according to the manufacturer's direction. Cells that had taken up the DNA were selected with 1 mg/ml G418 (Gibco BRL). Single colonies were picked 1 to 2

weeks after transfection and grown in medium containing 0.8 mg/ml G418. Colonies were screened for ability to take up fatty acids by measuring uptake of a fluorescently labeled fatty acid (BODIPY-FA) as described (Hirsch et al., 1998).

Northern Blotting. Human mRNA blots were obtained from Invitrogen. Blots were probed with ^{32}P -labeled DNA probes using the Rapid-Hyb buffer (Amersham) according to the manufacturer's instructions. Probes were generated by PCR from either the 3' untranslated regions or the poorly conserved 5' translated regions of human FATPs

In situ hybridization. Tissues were collected from 8-week-old C57/Bl6 mice. Tissues were fresh frozen, cut on a cryostat at 10 μm thickness and mounted on Superfrost Plus slides (VWR). Sections were air dried for 20 minutes and then incubated with ice-cold 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) for 10 minutes. Slides were washed twice for 5 minutes each with PBS, incubated with 0.25% acetic anhydride/1 M triethanolamine for 10 minutes, washed with PBS for 5 minutes, and dehydrated with 70%, 80%, 95% and 100% ethanol for 1 minute each. Sections were incubated with chloroform for 5 minutes. Hybridizations were performed with ^{35}S -radio-labeled (5×10^7 cpm/ml) cRNA probes generated from the 3' untranslated regions of mouse FATPs by PCR followed by in vitro transcription in the presence of 50% formamide, 10% dextran sulfate, 1x Denhardt's solution, 600 mM NaCl, 10 mM DTT, 0.25% SDS and 100 $\mu\text{g/ml}$ tRNA for 18 hours at 55°C. After hybridization, slides were washed with 10 mM Tris-HCl pH 7.6, 500 mM NaCl, 1 mM EDTA (TNE) for 10 minutes, incubated in 40 $\mu\text{g/ml}$ RNase A in TNE at 37°C for 30 minutes, washed in TNE for 10 minutes, incubated once in 2x SSC at 60°C for 1 hour, once in 0.2x SSC at 60°C for 1 hour, once in 0.2x SSC at 65°C for 1 hour and dehydrated with 50%, 70%, 80%, 95% and 100% ethanol. Localization of mRNA transcripts was detected by dipping slides in Kodak NBT-2 photoemulsion and exposing for 7 days at 4°C, followed by development with Kodak Dektol developer. Slides were counterstained with Haematoxylin and Eosin and photographed. Controls for the in situ hybridization experiments included the use of a sense probe that showed no signal above background in all cases.

Immuno-fluorescence and immuno-gold electron microscopy. Unfixed mouse small intestine was washed with Hank's buffered salt solution containing 1 mM EDTA, infused with 2.3M sucrose solution, and embedded in O.C.T., 4583 compound. The material was thick sectioned (15 μm - 40 μm). The sections were washed in PBS containing 1% BSA and 0.075% glycine to block non-specific binding. Primary and secondary antibodies were diluted in PBS with 10% FCS and incubated for one hour. The sections were mounted in 90% glycerol/PBS containing 1 mg/ml paraphenylenediamine, and examined with a BioRad MRC 600 confocal, mounted on a Zeiss Axioscop.

For the immuno-gold labeling, the tissue was fixed with 2% paraformaldehyde in PBS for 10 minutes, after which it was cryoprotected by infiltration with 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4. containing 20% polyvinylpyrrolidone, and then mounted on aluminum cryo nails and frozen in liquid nitrogen (Tokuyasu, K.T., 1986). Ultrathin sections were collected on carbon/formvar coated nickel grids. The primary antibody (anti-FATP4) was diluted in 10% FCS in PBS and incubated overnight at 4 C, followed by donkey anti-rabbit IgG-gold (12nm) (Jackson labs) for one hour. The sections were stained in 2% neutral uranyl acetate (20 minutes) and absorption stained with 2% uranyl acetate in 0.2% methylcellulose containing 3.2% poly-vinyl alcohol. The sectioned were examined with a Philips EM 410 electron microscope.

Enterocyte isolation and anti-sense oligonucleotide treatment. Enterocytes from male and female 2 to 8 month old BALB/c mice were isolated following standard procedures (Pinkus, 1981). In brief, small intestines (duodenum, ileum and jejunum) were removed, rinsed with Hanck's buffered salt solution (HBS, Gibco/BRL), cut into ~ 1 cm long sections and incubated in 50 ml HBS containing 0.1 M sucrose (BioRad) and 20 mM EDTA. Intestinal sections were gently stirred for 10 min. The detached enterocytes were filtered through sterile cheesecloth (VWR) and pelleted by centrifugation. The cells were then gently re-suspended in RPMI 1640 (GIBCO/BRL) containing 10% FCS and 0.01 mg/ml transferrin. Concentrated solutions of oligonucleotides were added to the enterocytes to yield the indicated final concentrations and the cells were incubated for 48 h at 37 C, 5% CO₂. Two anti-sense oligo-nucleotides with there respective controls were used showing comparable results. The sequences of the phosphothioate oligonucleotides were:

mmFATP4-S1 : GGAGCCTCTCTGGTGGGGG

mmFATP4-AS1: CCCCCACCAGAGAGGCTCC

mmFATP4-Control1: CCACCCCCGGAAAGCCTGC

mmFATP4-AS2: GGAGAACAGTAGCGCCCCAC

mmFATP4-Control2: GAGCCCGCCACCGTAGAGACA

LCFA uptake assays. Bodipy-FA uptake assays using FACS were performed as described previously (Hirsch et al. 1998) and also adapted to a 96 well format. LCFA uptake assays with enterocytes or with stably transfected 293 cells were done as follows. Mixed micelles of radio-labeled FA (NEN) and taurocholate (Sigma) in HBS were generated by brief sonication at 37 C. Equal volumes of cells and micelle solution were mixed, resulting in a final FA concentration of 25 μ M for anti-sense assays and 10 μ M for substrate specificity assays. Final taurocholate concentration was 5 mM. Cells were incubated for the indicated amount of time at 37 C. The assay was stopped by transferring the cells onto filter paper followed by extensive washes with ice cold HBS containing 0.1% BSA using a cell harvester (Brandell). Incorporated oleate was then determined by β -scintillation counting (Beckman).

Results

FATP4 is a functional fatty acid transporter

Full-length hsFATP1 and hsFATP4 cDNAs were identified by searching Millennium's databases using the BlastX algorithm (Altschul et al., 1990). A full-length mmFATP4 was amplified by PCR from a liver library. Alignments of human and mouse FATP1 and -4 showed 91% identity between hs- and mmFATP4 while the homologies to the closest related gene, FATP1, in the same species was significantly less (62% identity) clearly demonstrating that hsFATP4 is indeed the homologue of mmFATP4. During the preparation of this paper another group (Fitscher et al., 1998) independently cloned hsFATP4 and reported an amino acid sequence identical to ours.

Using a mammalian expression vector we generated 40 stable 293-cell lines expressing hsFATP4 and 20 cell lines transfected with a control plasmid. The ability of the different cell lines to take up FA, as assessed by uptake assays using the fluorescently labeled Bodipy-palmitate, correlated well with their FATP4 expression levels determined by Western blotting (Fig. 1A). All 20 vector control clones showed amounts of Bodipy-FA uptake similar to each other and to untransfected 293 cells. In contrast, among the 40 FATP4 transfected clones, a large number (~20) showed an approximately two-fold increase in Bodipy-FA uptake compared to any of the vector controls, and 3 had a 5 - to 10 fold increase in Bodipy-FA uptake.

Several of the cell lines with the highest amount of Bodipy-FA uptake as well as isolated primary enterocytes were used to measure the uptake of radiolabeled FAs. Short-term uptake by 293 cells and enterocytes of all FAs tested was linear (Fig. 1C). hsFATP4 expression enhanced the rate of palmitate uptake approximately 3 fold over 293 cells transfected with vector alone (Fig. 1C) and also accelerated the uptake of oleate but not of linolate, arachidonate, octanoate, butyrate or cholesterol (Table. 1). Isolated primary enterocytes showed a similar preference for palmitate and oleate, and absence of transport of arachidonate, octanoate, and butyrate, but displayed a more robust transport of linolate and cholesterol than the transfected 293 cells.

To further characterize the substrate specificity of FATP4 we measured the uptake by stably transfected 293 cells of 5 μ M Bodipy-FA in the presence of a 20 fold molar excess (i.e. 100 μ M) of FAs, FA-derivatives and lipid soluble vitamins and hormones. Both saturated and non-saturated fatty acids containing 10 to 26 C atoms strongly competed for uptake of Bodipy-palmitate (Figure 1B and Table 2) and thus are presumed to be substrates of FATP4. In contrast, fatty acids with 8 or fewer C atoms did not compete and thus are presumed not to be FATP4 substrates. Similarly, esters of long chain FAs and other hydrophobic molecules tested had no effect on uptake of Bodipy-palmitate.

FATP4 is strongly expressed by the enterocytes of the small intestine

To determine the cellular distribution of FATP4 in E18.5 C57/B16 embryos *in situ* hybridization with a non-crossreacting probe was used. On embryo whole mounts strong mmFATP4 expression could be seen in the small intestine (Fig. 2A, left panel). Microscopy of counter stained sections showed that FATP4 mRNA was present at high levels in the enterocytes of the intestinal villi (Fig. 2A, right panels). *In situ* hybridization of cross sections of the ileum, and duodenum from 8-week-old adult mice also demonstrated a strong expression of FATP4 in enterocytes (Fig. 2B). The highest expression levels of FATP4 were seen in the enterocytes of the jejunum and ileum, and lower, but significant, amounts were detected in the epithelial cells of the duodenum. However, FATP4 mRNA was undetectable in any other cell type of the small intestine such as mesenchymal cells, endothelial and smooth muscle cells and was completely absent from the colon (Fig 2B, left 6 panels). Interestingly, while the expression of FATP4 in the mature enterocytes of the villi was exceptionally high, it was low or undetectable in the undifferentiated precursor cells in the crypts between the villi (Fig 2A, right panel and 2B upper left 4 panels). No signals above background were detected for mmFATP1, mmFATP3 (data not shown) and mmFATP5 in any of the intestinal tissues (Fig. 2B, right 6 panels). mmFATP2 was detected by *in situ* hybridization at low levels in the epithelial cells of the ileum, jejunum and duodenum. Northern blot analysis of hsFATP1-6 in human ileum and jejunum confirmed the notion from the murine data that only FATP4 is expressed at appreciable levels in the small intestine (Fig. 3).

The FATP4 protein is localized to the apical side of enterocytes

To further characterize the FATP4 protein and its localization we raised a polyclonal anti-serum against a GST-fusion protein of the C-terminus of mmFATP4 in rabbits. The anti-serum showed only weak cross reactivity with the GST-fusion proteins of C-termini of other FATP family members in Western blot experiments (Fig. 4A). In Western blot experiments with lysates from isolated enterocytes from 3 different adult Balb C mice the anti-serum exclusively recognized a single ~70 kD band which is in accordance with the predicted molecular weight of 72 kD for mmFATP4 (Fig. 4B). This signal was specific for FATP4 since none was obtained with pre-immune serum (data not shown) and, more importantly, it could be abolished by pre-incubation of the anti-FATP4 serum with FATP4-GST fusion protein (Fig. 4B).

Immuno-fluorescence microscopy of fresh frozen un-fixed sections of adult mouse small intestine with the FATP4-specific anti-serum confirmed the expression of FATP4 in the epithelial cell layer of the villi (Fig. 5B) while incubation with a pre-immune serum from the same rabbit demonstrated only weak background fluorescence (Fig. 5A). At higher magnifications (Fig. 5C and D) it was apparent that FATP4 is preferentially localized to the apical side of the enterocyte which faces the lumen of the small intestine. Similar observations were made for the localization of hsFATP4 in the human ileum and jejunum (data not shown). Further

analysis of the sub-cellular localization of FATP4 in enterocytes by deconvolution microscopy confirmed that the transporter is localized to the apical side of the enterocyte including the brush border membrane (Fig. 5E).

Immuno-electron microscopy of fresh-frozen sections through the small intestine using a FATP4-specific polyclonal anti-serum showed a gradient of protein distribution starting from the apical side of the para-nuclear region and being most prominent in the micro-villi of the brush border membrane and unidentified membranous structures underlying this area (Fig. 6A). FATP4 molecules could be consistently detected in association with the plasma membrane of microvilli in the enterocyte brush border (Fig. 6B and C).

The apical localization of FATP4 and its exclusion from the baso-lateral face of the enterocyte is indicative of a role of the transporter in absorption of dietary fatty acids rather than the import of fatty acids from the blood.

FATP4 is required for efficient uptake of fatty acids by enterocytes. To demonstrate the importance of FATP4 for the absorption of dietary lipids by the small intestinal epithelium we developed an anti-sense based approach to modulate the expression of FATP4 in primary cultures of enterocytes. To this end mouse enterocytes were isolated from the small intestine and cultured *ex vivo* for 48h. Although cells did not reattach to the tissue culture treated plastic, viability after 2 days was usually greater than 90%. LCFA uptake was measured at 37 C by incubating enterocytes with a solution of mixed micelles consisting of radio-labeled FAs and the bile acid taurocholate, thereby mimicking the presentation of fatty acids in the intestine after the cleavage of triglycerides by pancreatic lipase. At a 50 μ M concentration, uptake of [3 H] oleate by cultured enterocytes was linear over a 10 min. time interval (Fig. 7A) and was temperature-dependent (data not shown). Incubation of enterocytes for 48h with a 100 μ M solution of a phosphothioate oligonucleotide corresponding to nucleotides 10 to 28 of mmFATP4 in the sense orientation had no effect on the rate of oleate uptake, when compared to untreated cells (Fig. 7A). However, incubation with the corresponding anti-sense oligonucleotide reduced the rate of enterocyte oleate uptake by ~50%. In contrast, a mismatched control oligonucleotide with identical base composition had no effect on FA uptake (Fig. 7B). The cell viability in all cases was comparable (~90%). FATP4 anti-sense treatment affected only FATP4 mediated uptake processes since two FATP4 anti-sense oligonucleotides (nucleotides 10 to 28 and 22 to 42) inhibited palmitate and oleate but not methionine uptake and incorporation (Fig. 7C). Furthermore, we evaluated the dose-response of anti-sense inhibition and its correlation with FATP4 protein levels. Incubation with increasing concentrations of the FATP4 anti-sense oligonucleotide resulted in increased reductions of [3 H] oleate uptake and a proportionate decrease in the level of FATP4 protein; at the highest concentration of oligonucleotide tested both were decreased by 60% (Fig. 7B). Thus, [3 H] oleate incorporation by enterocytes is proportional to the level of FATP4 protein, and we conclude that FATP4 accounts for most FA transport by isolated enterocytes.

Discussion

While intestinal transport systems for metabolites such as carbohydrates have been well characterized, the molecular mechanisms of fatty acid transport across the apical plasmalemma of enterocytes have remained largely unclear. Here we identify FATP4 as the principal fatty acid transporter in the small intestine that mediates the efficient uptake of dietary fatty acids.

FATP4 is expressed at high levels on the apical side of mature enterocytes in the small intestine. Stable over-expression of FATP4 in 293 cells significantly enhances the uptake of long chain fatty acids with the same specificity as enterocytes. FATP4 expression enhanced uptake of palmitate and, to a lesser extent, that of oleate, but not of FAs with fewer than 10 C atoms. Isolated enterocytes exhibited a similar preference for palmitate over other tested FAs and, identical to FATP4- expressing 293 cells, did not incorporate significant amounts of octanoate, arachidonate or butyrate. FATP4-specific uptake of the essential FA linolate by transfected 293 cells was significantly lower than that of enterocytes. However, uptake of linolate as well as oleate by control transfected 293 cells was higher than uptake of these FAs by enterocytes, potentially masking small increases in linolate and oleate uptake due to FATP4 expression. 293 cells are a kidney-derived carcinoma cell line, and kidney expresses high amounts of FATP2 and smaller levels of FATP1 (Hirsch et al., 1998). The expression of FATP1 and FATP 2 in 293 cells is currently unknown but could potentially be responsible for the observed differences between untransfected 293 cells and enterocytes.

Both competition and uptake assays demonstrate that FATP4 has a clearly defined substrate specificity and can mediate the transport of a wide range of physiologically relevant FAs. Short-chain FAs up to a chain length of C10 are not transported while saturated and un-saturated long-chain and very long-chain FAs are efficient substrates. Interestingly, arachidonate was inefficiently transported both by FATP4- expressing cell lines and enterocytes while stable expression of FATP1 in 3T3 cells did mediate a pronounced increase in arachidonate uptake (Schaffer and Lodish, 1994) hinting at distinct differences in substrate specificity between the different FATPs. Furthermore, while modifications at the end of the aliphatic chain, such as a Bodipy-group are tolerated, esterifications or other modifications of the carboxyl group led to an abrogation of transport.

Although FATP4 is present at low levels in a variety of tissues, most notably the brain (own unpublished results, Fitscher et al., 1998), it is the major FATP protein expressed in the small intestine as found by northern blot and *in situ* hybridization analysis. The epithelial cells of the ileum and the jejunum are the principle sites of dietary fatty acid absorption (Windler and Greten, 1989). FATP4 was expressed at high levels in the enterocytes in these locations as judged by *in situ* hybridization while it was absent from all other intestinal cell types. The notion that FATP4 is involved in the absorption of dietary fatty acids rather than solely the supply of an energy source to the enterocytes themselves was further supported by the observation

that FATP4 mRNA and protein levels were high in the differentiated enterocytes along the villi but low or absent in the undifferentiated progenitor cells in the crypts which have a high metabolic rate but are not involved in the absorption of nutrients. Furthermore, FATP4 was absent from the colon which is known to play only a marginal role in the absorption of long-chain fatty acids (Windler and Greten, 1989). Interestingly, our preliminary data show that a FATP isoform in *D. melanogaster* is localized in the mid- and hindgut of the larvae (data not shown) implicating FATPs also in nutritional uptake by invertebrates.

Nutritional transporters such as glucose transporters and other membrane proteins can be targeted to either of two distinct plasma membrane compartments in enterocytes (Rodriguez-Boulan and Nelson, 1989; Stevens, 1992). Apical transporters are involved in the uptake of nutrients from the lumen of the intestine and are often energy coupled. In contrast, baso-lateral transporters allow the absorbed molecules to leave the enterocytes and enter the blood stream, as is the case for sugars, while exocytosis at the baso-lateral face allows larger particles such as chylomicrons to leave the enterocyte and enter the lymphatics. We demonstrate here that FATP4 in enterocytes is found predominantly in the apical compartment, including the microvilli of the brush border membrane, suggesting that FATP4 is involved in the uptake of dietary fatty acids from the intestinal lumen into the enterocyte.

The fact that FATP4 is a functional long-chain fatty acid transporter highly expressed on the apical side of enterocytes in the small intestine strongly suggest that it is involved in the transporter mediated uptake of dietary fatty acids. However, the overall contribution of FATP4 to the absorption of dietary lipids could not be concluded from these data alone. Therefore we used an anti-sense oligonucleotide based approach to modulate FATP4 expression in primary enterocytes. Phosphothioate-modified oligonucleotides have been successfully employed to modulate expression of a number of genes in different cell types (Agrawal and Zhao, 1998; Gewirtz et al., 1998) including the down regulation of membrane transport proteins (Oberbauer et al., 1996). Primary enterocytes have been successfully isolated before from many sources including rat and hamster (Mircheff and van Corven, 1990; Pinkus, 1981; Velasco et al., 1986). After isolation, enterocytes demonstrate saturable and temperature dependent uptake of LCFA (Gore et al., 1994, our unpublished data). FATP4 anti-sense treatment of the enterocytes greatly reduced their ability to take up LCFA while the corresponding sense control oligonucleotide had no effect. Concentration-dependent reduction of the level of FATP4 protein expression by anti-sense oligonucleotides was closely correlated with a reduction in FA uptake. A 60% reduction in the level of FATP4 protein led to a corresponding 60% reduction in the rates of oleate and palmitate uptake, indicating that FATP4 is indeed responsible for the majority of long-chain FA uptake into enterocytes. The notion that FATP4 anti-sense oligonucleotides reduced LCFA uptake specifically by inhibiting FATP4 expression was further supported by the fact that a chemically identical control oligonucleotide with scrambled sequence had no effect and by the observation that other energy dependent uptake processes, such as amino-acid uptake, were not altered by the FATP4 anti-sense oligonucleotide

treatment. Furthermore, two different FATP4 anti-sense oligonucleotides, both immediately downstream of the translational initiation site, inhibited FA uptake into enterocytes while their respective mismatched controls had no effect. Three additional oligonucleotides further downstream of the FATP4 gene were also tested but showed only marginal (10 – 15%) inhibition of FA uptake; however in all cases incubation with the corresponding control mismatched oligonucleotides also had no inhibitory effect (data not shown). These data show, for the first time, that the down-modulation of an endogenous FATP in primary cells results in a significantly reduced rate of FA uptake, illustrating the importance of FATPs for this process.

Besides the FATP family several other molecules have been implicated in the binding and transport of fatty acids. A homologue of the human CD 36 scavenger receptor (FAT) binds a large number of hydrophobic molecules including fatty acids (Baillie et al., 1996) and is localized to adipocytes, myocytes, mammary cells and enterocytes (Sfeir et al., 1997). However, it is absent from liver but highly expressed in organs not associated with FA metabolism such as spleen (Abumrad et al., 1993). Plasma membrane fatty acid binding protein (FABPpm), a 43 kD molecule, transiently increases fatty acid uptake in 3T3 fibroblasts and is expressed in several tissues including heart, liver and small intestine (Sorrentino et al., 1988; Stremmel et al., 1985; Stremmel et al., 1985). Furthermore, antibodies against FABPpm were reported to decrease oleate uptake of perfused jejunal segments (Stremmel, 1988). However, FABPpm was subsequently identified as the mitochondrial isoform of aspartate aminotransferase (Stump et al., 1993) and it remains unclear how this mitochondrial enzyme could participate in the transport of fatty acids across the plasma membrane. FATP4 expression correlates closely with FA uptake in 293 cells and enterocytes and FATP4 can mediate the uptake of all physiological long-chain FAs tested, demonstrating that FATP4 is the major FA transporter in the small intestine. However it is likely that efficient FA uptake requires many components upstream as well as downstream of FATPs, possibly including extracellular scavenger receptors such as CD36, and intracellular molecules like LCFA CoA-ligases and fatty acid binding proteins.

Over 55 million people in the US alone suffer from obesity (Van Itallie, 1996) contributing significantly to obesity related health problems such as hypertension and type-2 diabetes (Ernst et al., 1997). Lipids in the average western diet contribute up to 40% of total calories and efforts have been made to reduce the caloric input either by substitution of lipids with non-digestible analogs (Lawson et al., 1997) or by inhibiting pancreatic lipase (Guercioli, 1997). Since enterocytes can only absorb free fatty acids but not tri-glycerides, inhibition of hydrolysis of lipids by lipases blocks their uptake. However, recent clinical studies have only shown a modest effect of such treatments (Davidson et al., 1999). An alternative or additional treatment could be direct inhibition of fatty acid uptake (Thomson et al., 1997) which could be theoretically achieved by blocking FATP4 function. Our anti-sense data have shown that such treatments could in principle result in drastic reduction of LCFA uptake by the small intestine, making FATP4 an attractive target for future anti-obesity drugs.

Figure and Table Legends

Table 1. Uptake of different substrates by enterocytes and by control- and stable FATP4- expressing 293 cells.

The rates of uptake for the indicated fatty acids was measured over 4 min. taking measurements every 30 sec. All fatty acids were at a concentration of 10 μ M in HBS containing 5 mM taurocholate.

Table 2. Competition for bodipy-FA uptake by FATP4 expressing cells by different hydrophobic compounds

The uptake of 5 μ M Bodipy-FA, C1-Bodipy-C12, was measured in the presence a 20 fold molar excess (i.e. 100 μ M) of the indicated fatty acids or fatty acid derivatives. The maximal 100% inhibition was defined as the amount of Bodipy-FA incorporated in the presence of 200 μ M lauric acid which was on average $18 \pm 5\%$ that of untreated cells.

- 0 – 30% inhibition by the indicated substance.
- \pm 30 – 50% inhibition,
- + 50 – 70% inhibition
- ++ 70 – 100% inhibition.

Figure 1. FATP4 mediates LCFA uptake

(A) 293 cells were stably transfected with mammalian expression constructs containing either hsFATP4 (squares and triangles) or empty control vector (circles). Short-term uptake of Bodipy-FA in the presence of BSA was determined by FACS. The mean fluorescence of the viable cell population is expressed in arbitrary fluorescence units. FATP4 protein expression was determined by densitometry of anti-FATP4 western blots, shown in the insert, and is expressed in arbitrary units.

(B) Short-term uptake of Bodipy-palmitate (1 μ M) either by control cells (black bars) or FATP4 expressing cells (hatched bars) was measured in the presence of 0, 10 or 100 μ M unlabeled palmitate. FA uptake was quantified by FACS and expressed in arbitrary fluorescence units.

(C) 293 cells were stably transfected with a construct for either human FATP4 (diamonds) or an empty vector (circles) and their rate of [3 H] palmitate uptake was compared to that of isolated enterocytes (squares).

Figure 2. FATP4 is localized in enterocytes by *in situ* hybridization

(A) *In situ* hybridization of a section through a whole E18.5 embryo (left panel) and sections through the small intestine of E18.5 embryo (right panels) with a FATP4 specific ribo-probe shown at 200x in phase contrast and dark field.

(B) *In situ* hybridization of a section through duodenum, ileum, and colon of adult mice with FATP4 and FATP5 specific ribo-probes shown at 200x in dark field and phase contrast.

Figure 3. FATP4 is the prevalent member of the FATP family in the ileum and jejunum.

Northern blot analysis of the expression patterns of hsFATP1 through 6 in the ileum and jejunum. Probes were from unique region of the genes and did not cross-react with other FATP members. As a control all probes were hybridized under similar conditions to mRNAs from a variety of FATP expressing tissues. FATP1: heart, FATP2: kidney, FATP3: lung, FATP5: liver, FATP6: heart.

Figure 4. The FATP4 protein is specifically detected by a polyclonal anti-serum.

(A) Western blot of GST-fusion proteins of the C-termini of FATP2 through 5 with a polyclonal anti-serum against the C-terminus of FATP4.

(B) Western blot detection of FATP4 in enterocyte lysates from 3 different mice without or with pre-incubation of the anti-serum with the antigen. Molecular mass standards are indicated in kD.

Figure 5. FATP4 is located on the apical face of enterocytes.

Fluorescent and phase-contrast pictures of murine small intestine. Fresh-frozen thin sections were either incubated with pre-immune (A) or anti-FATP4 serum (B).

Confocal laser microscopy images of FATP4 in the small intestine at 20x (C) and 63x (D). Deconvolution microscopy of a small intestinal thin section (E) stained with anti-FATP4 (green) and DAPI (blue) at a 40x magnification.

Figure 6. FATP4 is present in the microvilli.

(A-C) Immuno-electron microscopy of fresh-frozen murine intestinal cells.

Fresh-frozen unfixed micro sections of murine ileum were incubated with FATP4 specific anti-serum, which was detected by 10 nm gold conjugated secondary antibodies. Longitudinal section through brush border

membrane (A, bar = 0.21 μ M). Cross-section through microvilli (B, bar = 0.3 μ M); arrows indicate the position of the gold particles. High magnification cross-section through micro villi (C, bar = 0.12 μ M).

Figure 7. FATP4 is required for efficient LCFA uptake by enterocytes

(A) The kinetics of [3 H] oleate incorporation by enterocytes isolated from the small intestine of mice were measured after incubation for 48h in tissue culture either without oligonucleotides (squares) or with 100 μ M FATP4- specific sense (circles) or anti-sense (diamonds) oligonucleotides.

(B) Isolated enterocytes were incubated for 48 hours with increasing concentrations of the FATP4 anti-sense oligonucleotide or with 100 μ M of a randomized control oligonucleotide with identical nucleotide composition to the FATP4 anti-sense oligonucleotide. The uptake of oleate by the enterocytes was then measured over a 5 min time interval (solid bars). In parallel, the levels of FATP4 protein and, as a loading control, β -catenin, were determined by Western blotting and quantitated using densitometry (hatched bars). FA uptake and FATP4 protein levels were normalized to that of untreated cells. The averages and standard deviations of 4 independent experiments are shown.

(C) Uptake rates of [3 H] oleate, [3 H] palmitate and [35 S] methionine by primary enterocytes were measured after 48h incubation with either of two FATP4 anti-sense (solid bars) or randomized control oligonucleotide (hatched bars) at a concentration of 100 μ M and expressed as % of untreated cells.

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Fig 1

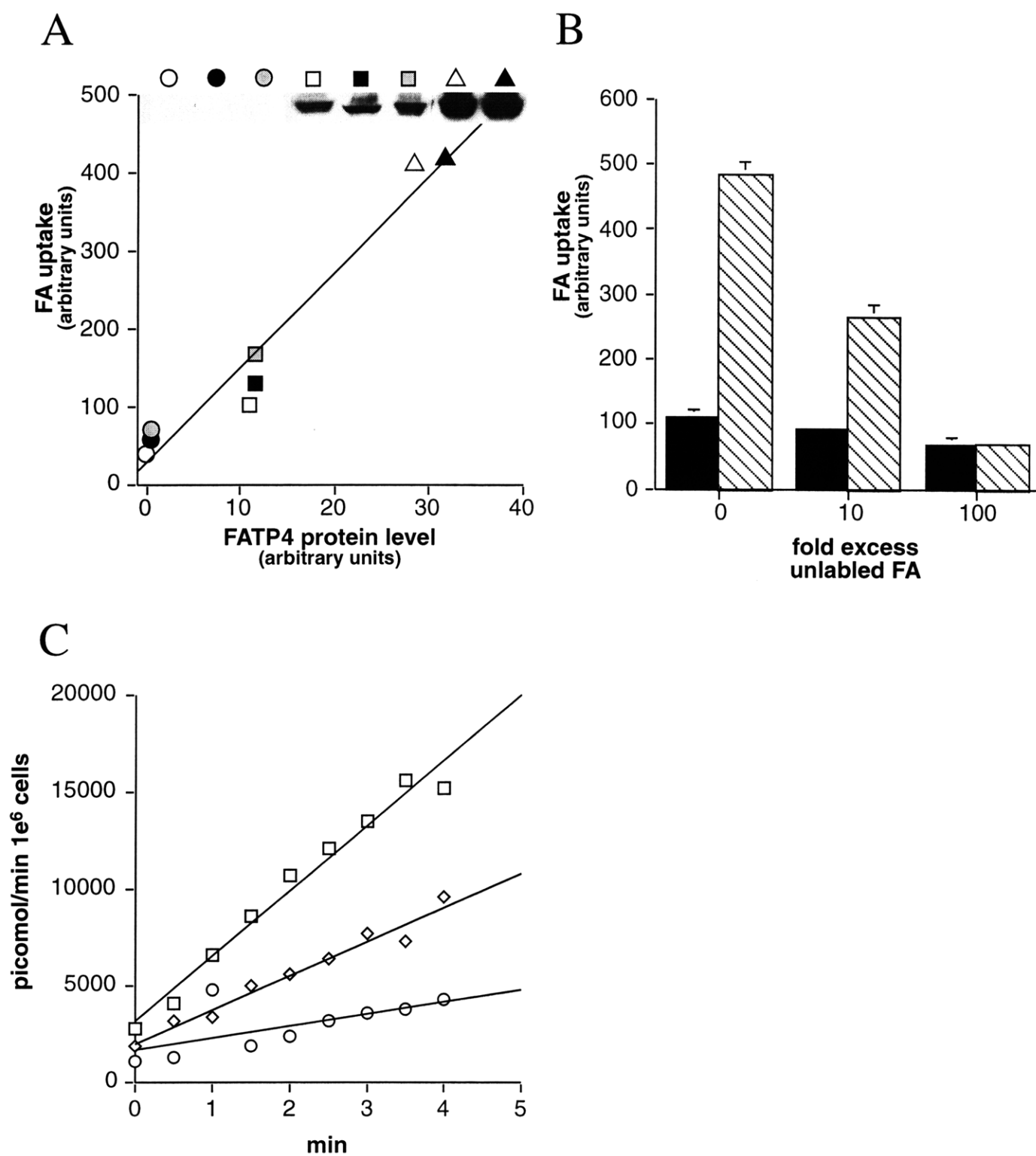


Table 1. Uptake of different substrates by FATP4 expressing cell lines and enterocytes

Fatty Acid	Control*	<u>293 Cells</u> Stably expressing FATP4	FATP4 specific	Enterocytes*
Palmitate	564	1695	1131	3036
Oleate	662	1122	459	117
Linolate	640	673	33	116
Arachidonate	3	5	2	0
Octanoate	0	0	0	5
Butyrate	0	50	50	73
Cholesterol	319	345	26	531

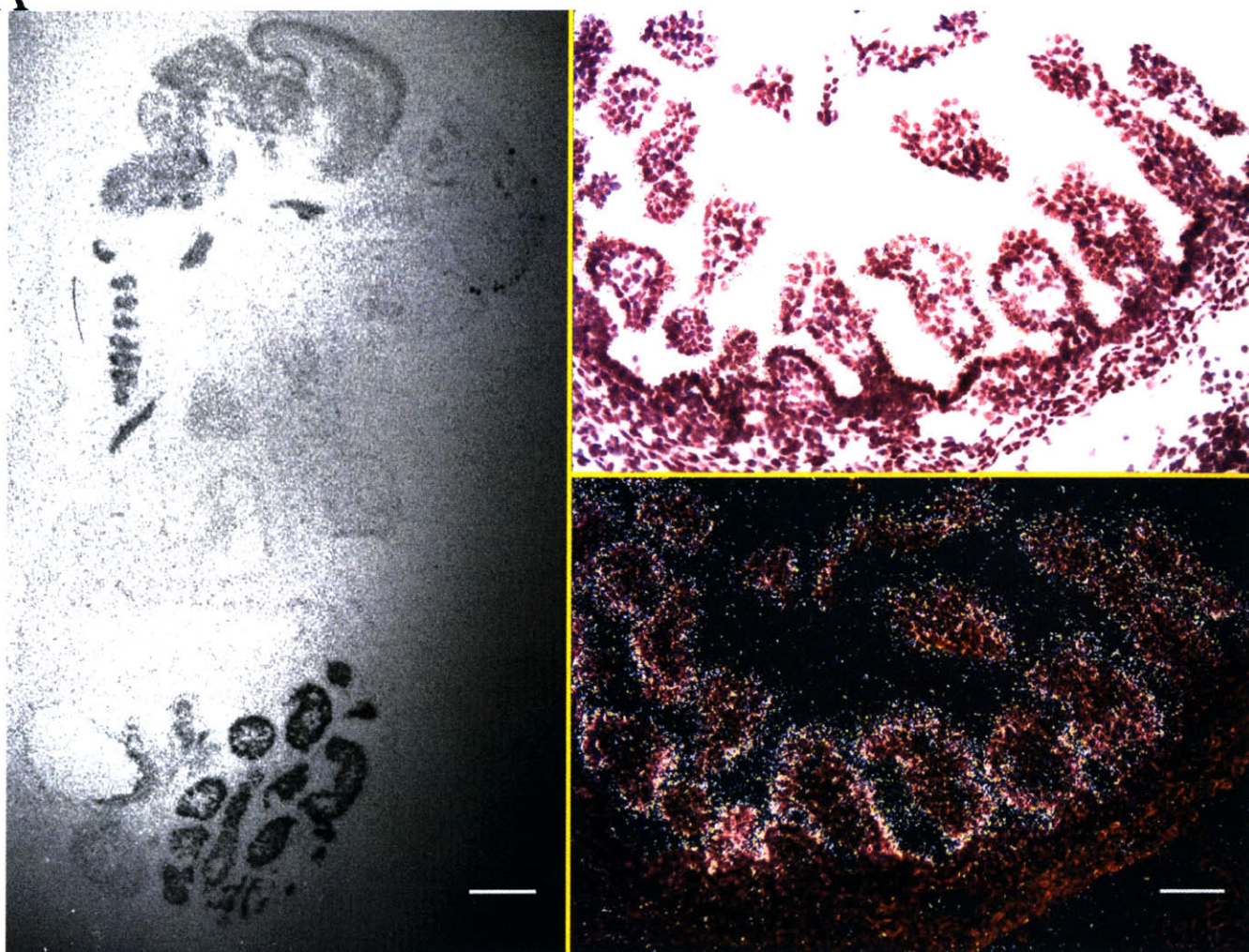
* Uptake measured as *pmol/min 10⁶ cells*

Table 2. Competition of Bodipy-FA uptake by FATP4 expressing cells

<i>Fatty Acids</i>	<i>Formula</i>	<i>Competition</i>
Butyric Acid	C ₄ H ₈ O ₂	-
Caproic Acid	C ₆ H ₁₂ O ₂	-
Caprylic Acid	C ₈ H ₁₆ O ₂	-
Capric Acid	C ₁₀ H ₂₀ O ₂	++
Lauric Acid	C ₁₂ H ₂₄ O ₂	++
Myristic Acid	C ₁₄ H ₂₈ O ₂	++
Palmitic Acid	C ₁₆ H ₃₂ O ₂	++
Stearic Acid	C ₁₈ H ₃₆ O ₂	+
Oleic Acid	C ₁₈ H ₃₄ O ₂	++
Linoleic Acid	C ₁₈ H ₃₂ O ₂	++
Arachidic Acid	C ₂₀ H ₄₀ O ₂	++
Lignoceric Acid	C ₂₄ H ₄₈ O ₂	++
Cerotic Acid	C ₂₆ H ₅₂ O ₂	++
<i>Fatty Acid Derivatives</i>		
Palmitic Acid Methyl Ester	C ₁₇ H ₃₄ O ₂	-
Stearic Acid Methyl Ester	C ₁₉ H ₃₈ O ₂	-
Oleic Acid Ethyl Ester	C ₂₀ H ₃₈ O ₂	-
Oleic Acid Oley Ester	C ₃₆ H ₆₈ O ₂	-
Oleoyl CoA	C ₃₉ H ₆₈ N ₇ O ₁₇ P ₃ S	-
Cholesteryl Oleate	C ₄₅ H ₇₈ O ₂	-
<i>Lipid-Sol. Vitamins & Hormons</i>		
Retinoic Acid (Pro-Vitamin A)	C ₂₀ H ₂₈ O ₂	±
Ergocalciferol (Vitamin D2)	C ₂₈ H ₄₄ O	-
Tocopherol (Vitamin E)	C ₂₉ H ₅₀ O ₂	-
3-Phytylmenadione (Vitamin K1)	C ₃₁ H ₄₆ O ₂	-
Prostaglandin E2	C ₂₀ H ₃₂ O ₅	-

Fig 2

A



B

FATP4

FATP5

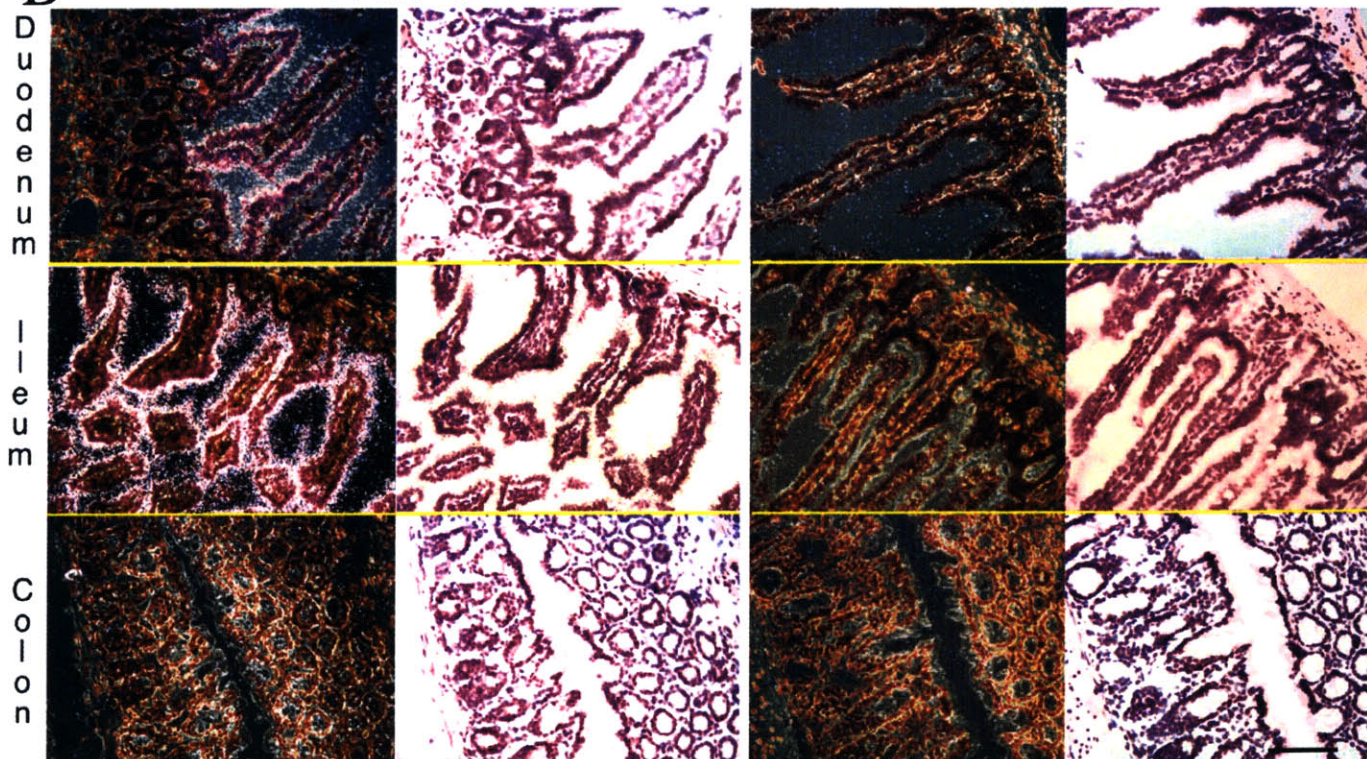


Fig 3

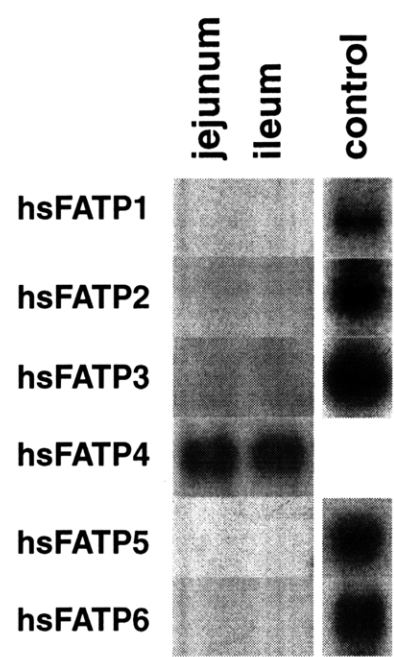


Fig 4

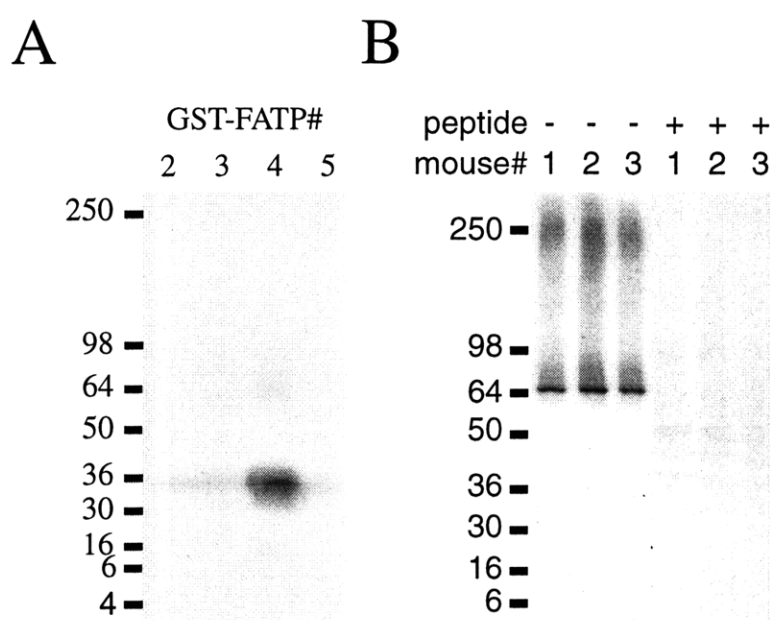


Fig 5

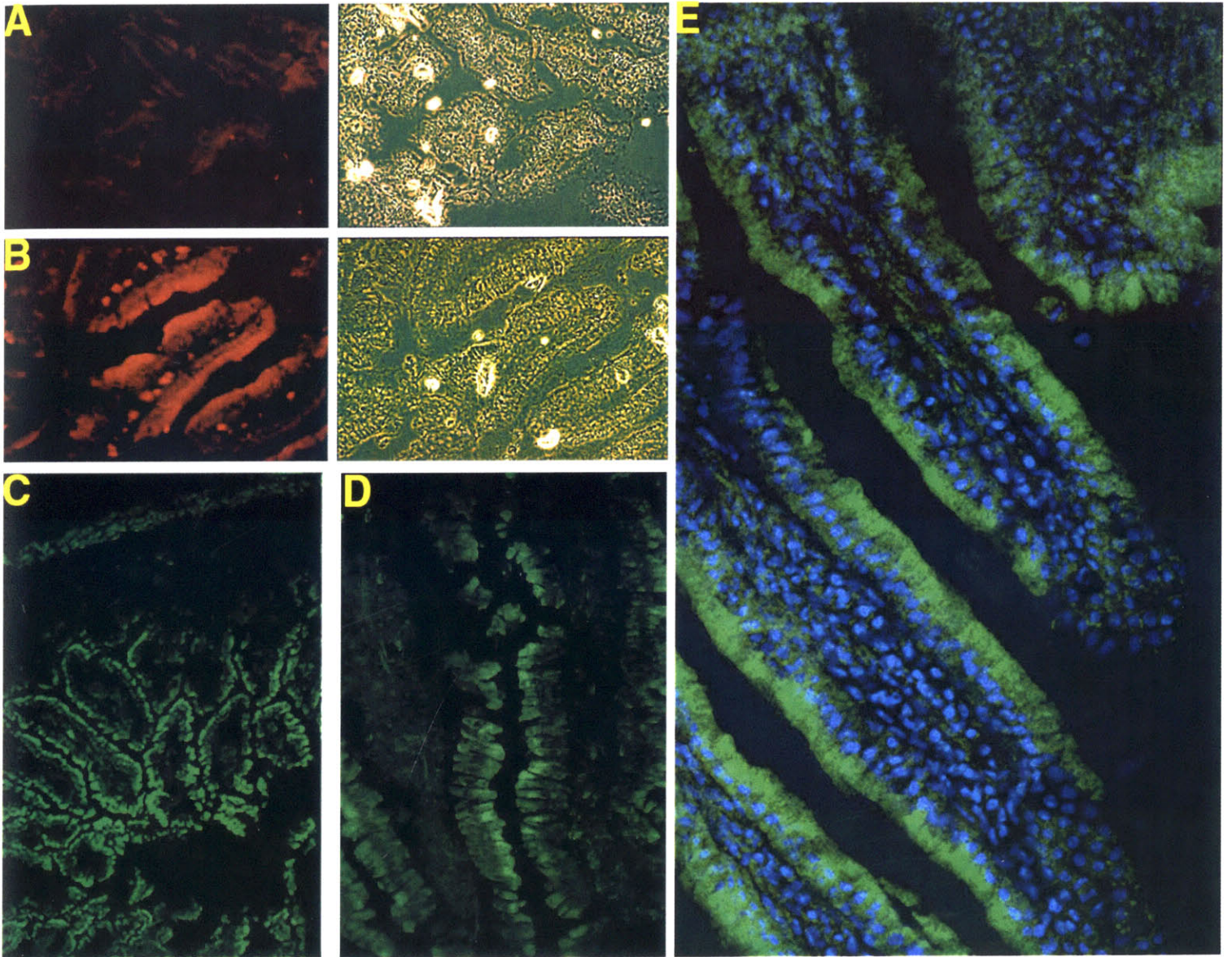


Fig 6

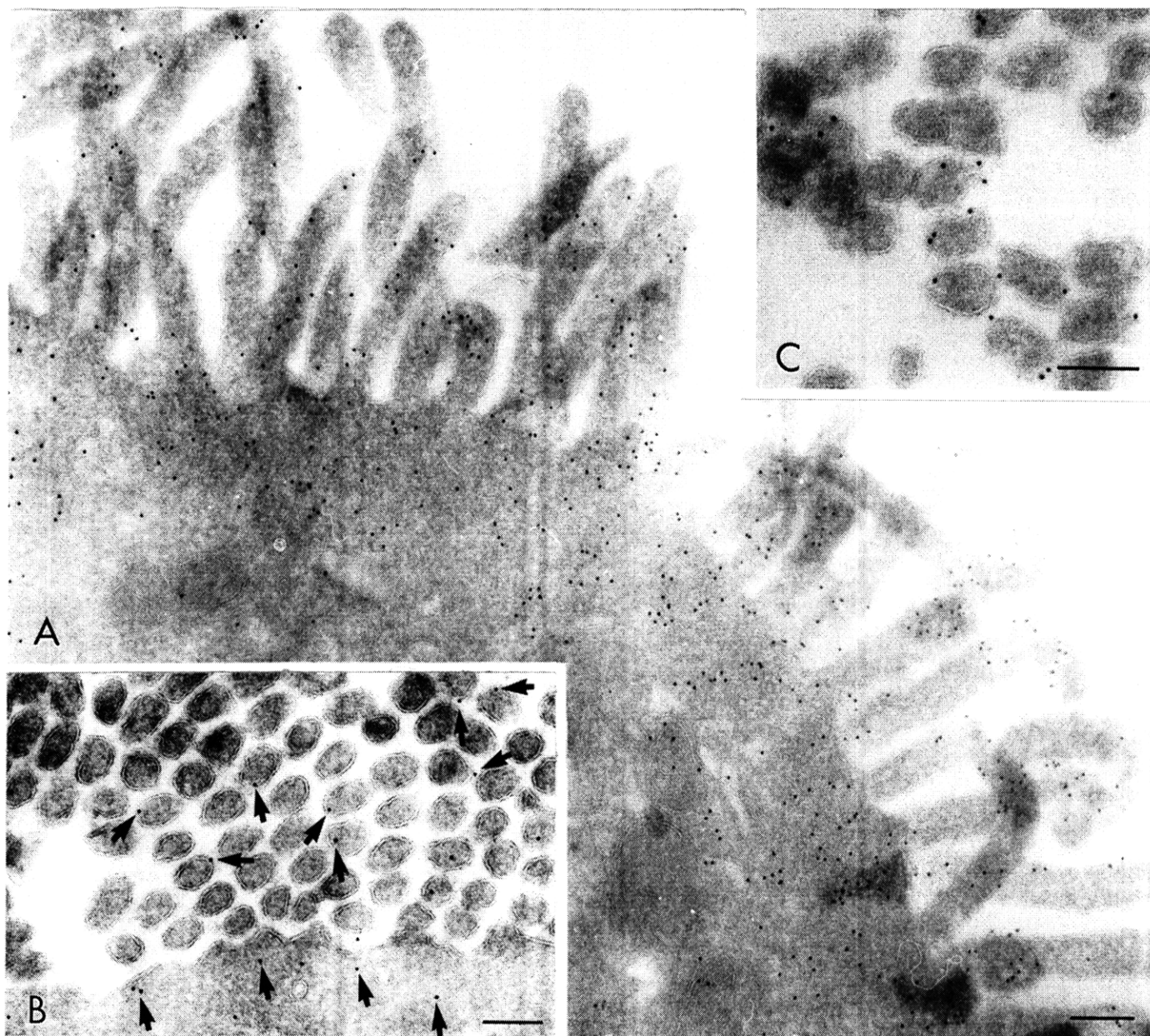
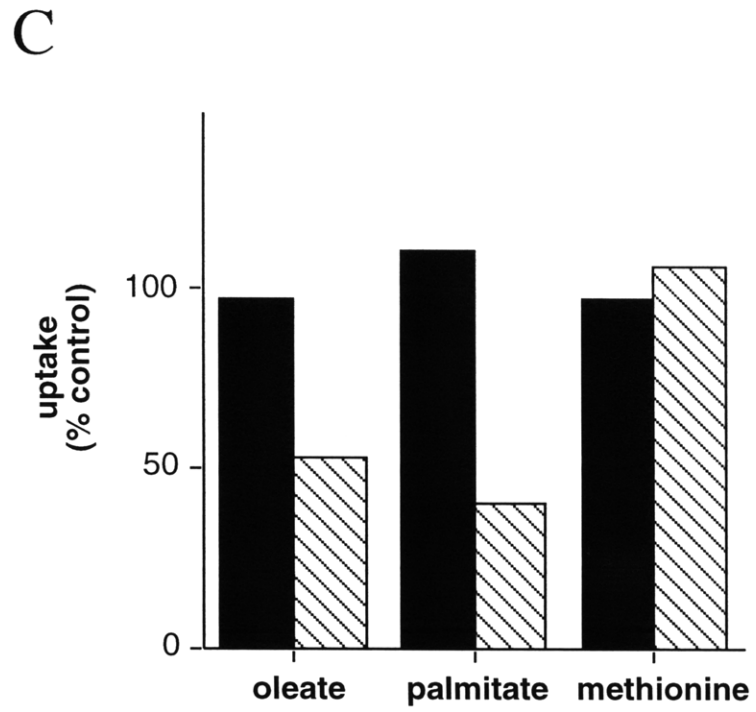
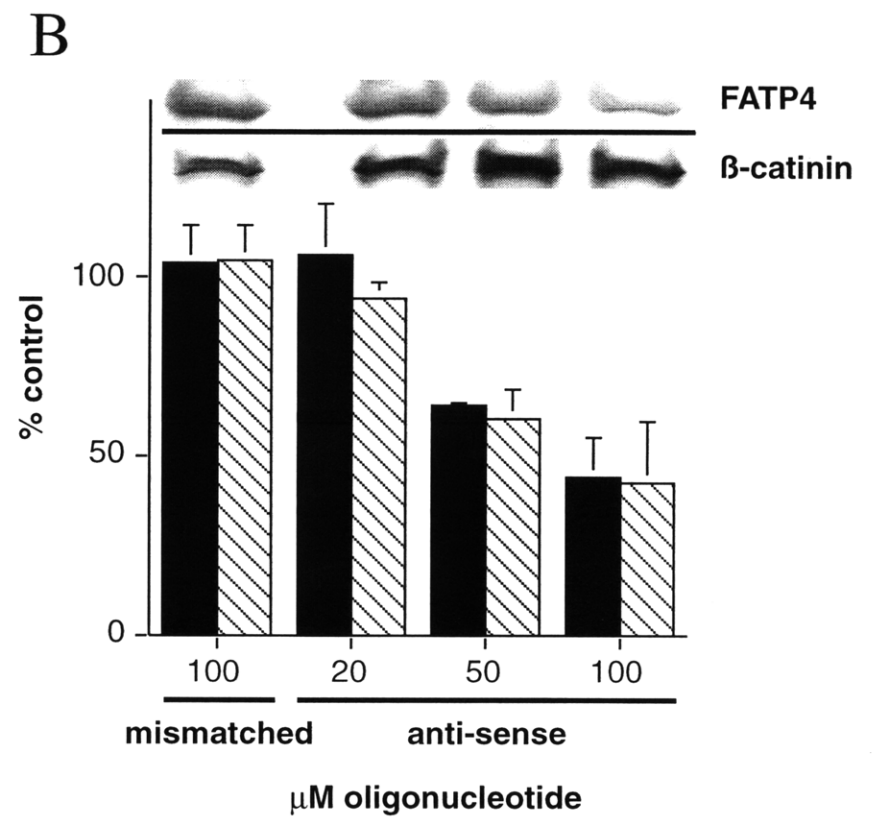
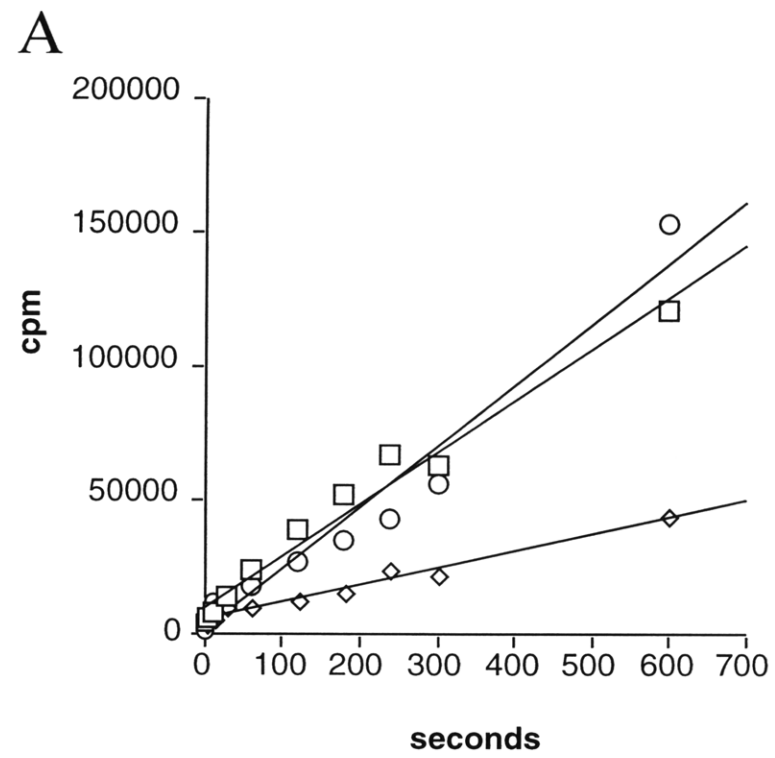


Fig 7



Chapter 5

Identification and characterization of the FATP5 promoter

Abstract

Long chain fatty acids (LCFA) are an important source of energy for most organisms. They also function as blood hormones, regulating key metabolic functions such as hepatic glucose production. While LCFAs can diffuse through the hydrophobic core of the plasma membrane into cells, such non-specific transport may not account for the high affinity and specific transport of LCFAs exhibited by cells such as cardiac muscle, hepatocytes and adipocytes (Abumrad et al., 1999; Berk and Stump, 1999). Transport of LCFAs across the plasma membrane may be mediated by members of the FATP gene family. There are six mammalian FATPs which are expressed in a highly tissue specific manner (Hirsch et al., 1998). Interestingly, FATP5 expression in the mouse was confined to the liver, which is known to have a large capacity for fatty acid uptake. To study the molecular basis for its tissue specific expression, we identified the cis-acting elements in the mouse FATP5 promoter. We isolated a BAC encoding the genomic FATP5 locus and subcloned 8 kb of genomic DNA upstream of the initiator methionine into a luciferase reporter vector. When transfected into cell lines of different origins, the 8 kb

FATP5 promoter was active only in liver cell lines. Deletion analysis of the 8 kb genomic DNA revealed that tissue specific promoter activity was retained in constructs containing 261 base pairs but not 218 base pairs upstream of the initiator methionine. The sequence between -261 and -218 contained a single GC box and mutating this sequence abolished promoter activity. Gel shifts using an oligonucleotide containing this GC box formed multiple complexes with HepG2 nuclear extracts. However, nuclear extracts from a non-liver cell line in which the FATP5 promoter was inactive yielded the same pattern suggesting that other cis-acting promoter elements might exist. We identified two additional motifs downstream of the GC box essential for FATP5 promoter activity. These motifs did not match the consensus binding sites of any known transcription factor suggesting that novel proteins may be involved in the transcription of FATP5. Elucidation of the cis-acting elements required for FATP5 transcription may provide insight into the physiologic regulation of this gene and its role in lipid metabolism.

Introduction

Many mammalian tissues have a robust ability to take up fatty acids from the serum. First pass clearance of fatty acids from the blood is greater than 50% for both heart and liver (Glatz et al., 1997; Stremmel, 1989). Additionally, fatty acid uptake by adipocytes is crucial for the storage of energy. For many years, fatty acid transport across the plasma membrane was thought to be mediated by simple diffusion (Hamilton, 1998). However, over the past 15 years, convincing evidence that plasma membrane proteins facilitate the transport of fatty acids across the plasma membrane has emerged (Abumrad et al., 1999; Berk et al., 1996; Berk and Stump, 1999; Fitscher et al., 1996; Glatz et al., 1997; Schaffer and Lodish, 1995). FATP, now called FATP1, was cloned by Schaffer et

al. using an expression cloning strategy to identify cDNA's which enhanced fatty acid uptake when over-expressed in Cos cells (Schaffer and Lodish, 1994). FATP specifically increased uptake of long chain fatty acids but not medium chain fatty acids or structurally unrelated substances such as glucose or cholesterol. Subsequently, it was found that FATP was part of a large protein family (Hirsch et al., 1998). In addition to six human and five mouse genes, FATPs were identified in such diverse organisms as *F. rubripes*, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, and *M. tuberculosis*. The function of the FATP gene family is conserved throughout evolution as both the *C. elegans* and *M. tuberculosis* FATPs facilitated fatty acid uptake when over-expressed in mammalian cells and *E. coli* respectively.

The presence of six FATP homologues in mammals suggests that FATPs may differ in their biochemical properties or patterns of expression. Interestingly, several of the FATPs have very discrete localizations. mmFATP4 is highly expressed in the enterocytes which line the small intestine and probably mediates the uptake of dietary fatty acids by these cells (Stahl et al., 1999). mmFATP2 (mus musculus) is only expressed in the liver and kidney, and within the kidney, FATP2 mRNA is restricted to the cortex (D. Hirsch, unpublished data). hsFATP6 is heart specific (Stahl et al., manuscript in preparation) and FATP5 is liver specific (Hirsch et al., 1998). Since the liver has a tremendous ability to take up fatty acids and plays a central role in lipid metabolism, we studied the FATP5 promoter to determine the molecular mechanisms for liver specific expression. Identification of these promoter elements may also provide insight into the physiologic regulation of FATP5 and its role in mammalian physiology.

Methods

BAC Isolation and Luciferase Constructs

An arrayed BAC library was screened by PCR for FATP5 genomic clones. PCR primers designed by a program from the Whitehead Institute's Genome Center specifically amplified a single band of the correct size from mouse genomic DNA. Two putative BACs containing the FATP5 genomic sequence were identified and the presence of FATP5 sequence was confirmed by dot hybridization of the BAC with the mmFATP5 cDNA.

After isolation of positive BACs, large amounts of bacteria were grown and DNA prepared using a Qiagen maxi-prep kit (Qiagen). The BAC was digested with Sac I and ligated into pZero-2 (Invitrogen). Inserts containing mmFATP5 genomic sequence were identified by screening colony lifts of the ligation with an α -³²P-ATP radiolabeled, random primed (Boehringer-Mannheim) mmFATP5 cDNA as a probe. Positive colonies were picked and restriction analysis with Sac I revealed them to contain an identical, large insert of 8-10 kb. Digestion of the Sac I fragment with BstX I yielded three pieces that were subsequently subcloned into pZero and sequenced using an ABI sequencer (Research Genetics). A 1.3 kb piece containing sequence immediately upstream of the FATP5 initiator methionine was subcloned into the Xho I and Bgl II sites of the promoter-less pGL3 luciferase reporter vector (Promega). 7 kb of additional upstream sequence was subcloned into the Xho I and Sac I sites of the prior construct to yield a final construct containing approximately 8 kb of genomic sequence upstream of the initiator methionine. Deletions of the FATP5 promoter were constructed using PCR with the 1.3 kb promoter construct as the template. Products were amplified with primers containing Hind III (5' primer) and Xho I (3' primer) sites using Elongase (Gibco). The resulting fragments were cut with Hind III and Xho I and subcloned into the

corresponding sites of the promoter-less pGL3 luciferase reporter vector. The internal 30 base pair deletions, GC box mutations, and 10 nucleotide linker scan were all created with the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's instructions. At least two different bacterial colonies were picked for each construct. The inserts from both colonies were sequenced to check for unintended point mutations and both constructs were assayed for luciferase activity.

Cell culture, Transfection, and Luciferase Measurements

HepG2, Hep3b, HT1080, 3T3-L1, Bosc, and HACAT cells were grown in DMEM supplemented with 10% fetal calf serum, 1 x penicillin-streptomycin and glutamine (Gibco). Mink lung cells were grown in MEM supplemented with 10% fetal calf serum, 1 x minimal essential amino acids, 1 x penicillin-streptomycin and glutamine. The evening prior to transfection, cells were plated at 50-60% confluence in 24 well dishes. The following morning, cells were placed in 2 mls of fresh media and 250 uL of a CaPO_4 solution (Invitrogen) containing 2 ug of a luciferase reporter construct and 0.5 ug of pCMV- β -gal was added to the cells. pCMV- β -gal constitutively expresses β -galactosidase and was used to normalize transfection efficiency (Hua et al., 1998). After 12 hours, the cells were washed twice with DMEM and placed in fresh media. Thirty six hours later, the media over the cells was removed and 250 uL of 1 x reporter lysis buffer (Promega) was added. After vigorous shaking for 15 minutes at room temperature, the supernatants were transferred to Eppendorf tubes and briefly centrifuged to remove particulates. 20 uL from these tubes was used for determination of luciferase activity (Promega) and 20 uL was used for the measurement of β -galactosidase activity (Clontech). All luciferase values were normalized to β -galactosidase to control for transfection efficiency and expressed as relative luciferase units (RLU). For experiments

comparing different cell lines, promoter activity was computed as a fold induction by dividing the RLU activity of either the -8 kb or -271 promoter constructs by the RLU activity a promoter-less construct. Each data point was done in triplicate and each experiment was repeated a minimum of three times.

Northern Blots, Preparation of Nuclear Extracts, and Gel Shift Assays

Human poly-A northern blots were purchased from a commercial vendor (Clontech) and probed with a piece of the human FATP5 3' untranslated region specific for FATP5. Nuclear lysates from HepG2 and Bosc cells were essentially prepared according to the method of Hua et al. and stored at -80°C (Hua et al., 1998). Probes for gel shift assays were end labeled using T4 polynucleotide kinase (Boehringer-Mannheim) and gel purified. Gel shifts were performed at room temperature in 30 uL reactions comprised of 6 uL 5 X binding buffer (100 mM Tris 8.0, 300 mM KCl, 5 mM EDTA, 8 mM MgCl₂, and 36% glycerol), 0.5 uL of 100 mM DTT, 1 uL of 10 mg/ml BSA, 2 uL of 2 mg/ml poly dI/dC, and 5 uL nuclear lysate. 10 minutes after the addition of nuclear lysate, 40,000 cpm of ³²P-labeled probe were added. After 20 minutes at room temperature, loading dye was added and the reaction run on a 4% non-denaturing gel.

Results

Human FATP5 mRNA is only expressed in adult liver

We had previously reported that mmFATP5 mRNA was only expressed in the liver (Hirsch et al., 1998). To determine if the human isoform of FATP5 was also liver specific, we performed northern analysis using a probe from the 3' transcribed but

untranslated region of the human gene. Similar to the mouse homolog, hsFATP5 is liver specific (Fig 1). Interestingly, hsFATP5 was not expressed in fetal liver suggesting that it may be developmentally regulated.

Identification of a FATP5 promoter

We next set out to determine the cis-acting elements responsible for liver-specific expression of FATP5. We identified BACs containing the FATP5 genomic locus and subcloned a 10 kb Sac I fragment which was subsequently sequenced. The Sac I fragment contains approximately 8 kb of genomic sequence upstream of the FATP5 initiator methionine. Blast searches using the 5' end of the Sac I sequence revealed that it contained coding sequence for an unknown gene immediately upstream of FATP5. Since the FATP5 promoter is unlikely to overlap the coding sequence of another gene, we hypothesized that the 10 kb Sac I fragment contained the FATP5 promoter. To test this hypothesis, 8 kb of genomic DNA upstream of the translational initiator of FATP was subcloned into the promoter-less pGL3 luciferase reporter vector. This construct was transiently transfected into the HepG2 liver cell line and luciferase activity was determined. The -8 kb piece of DNA resulted in a 35 fold induction of luciferase activity when compared to a pGL3 vector without the FATP5 genomic sequence (Fig 2). To determine if this activity reflected tissue specific transcription, the -8 kb luciferase reporter construct was transfected into a variety of additional cell types. While promoter activity was robust in the Hep3b hepatoma cell line, non-liver cell lines did not express large amounts of luciferase. Thus, the 8 kb upstream genomic element recapitulated liver-specific expression *in vitro*.

The FATP5 promoter resides within the 261 base pairs upstream of the initiator methionine and requires a single GC box

To determine the cis-acting elements in the -8 kb of genomic sequence responsible for transcriptional activity, serial 5' deletions of the promoter were constructed and transfected into HepG2 cells. Surprisingly, greater than 90% of the -8 kb was dispensable for promoter activity. A construct containing only 261 base pairs upstream of the initiator methionine resulted in promoter activity equivalent to that of the -8 kb construct (Fig 3). Identical results were obtained when the deletion series was transfected into Hep3b cells (data not shown). We next determined if promoter activity of a small genetic element was tissue specific. Transfection of a construct containing 271 base pairs upstream of the initiator methionine into a variety of cell lines essentially replicated the results of the -8 kb construct in that high levels of luciferase expression were observed only in liver derived cell lines (Fig 4). Although the HT1080 cell line had significant levels of luciferase activity, the amount was much less than that observed in the liver cell lines.

Since deletion analysis revealed that bases between -261 and -218 were required for promoter activity, we closely examined this region for binding sites of known transcription factors and found the sequence GGGGCGGGG between nucleotides -241 and -232 (Fig 5a). This sequence binds the Sp1 family of transcription factors and is termed a GC box. To determine if the activity of the -271 construct required the GC box, we mutated the GC box. The first construct deleted nucleotides -241 to -222 which removed the GC box and additional downstream sequence which, although less optimal, might also bind the Sp1 family of transcription factors. The second construct had three G to A point mutations in the GC box between nucleotides -241 to -232. Such mutations

had previously been shown to abolish transcriptional activity of GC boxes (Rodenburg et al., 1997). In contrast to the wild type -271 promoter, both of the mutated constructs were transcriptionally inactive in HepG2 cells (Fig 5b). Identical results were also obtained in Hep3b cells (data not shown). This suggests that the GC box between -241 to -232 is essential for transcriptional activity of the FATP5 promoter. We next examined whether the sequences necessary for luciferase activity also bound proteins in nuclear extracts from HepG2 cells. Two different oligonucleotides were used for gel shift analysis. One oligonucleotide (AF-1) contained nucleotides -250 to -230 and the other (AF-2) spanned nucleotides -260 to -200 (Fig 6a). Both oligonucleotides yielded three significant complexes from HepG2 nuclear extracts (arrows in Fig 6b). All complexes were specific as 100 fold excess of the same unlabeled oligonucleotide could compete for binding of the radiolabeled oligonucleotide. Mutant AF-1 oligonucleotides containing three point mutations in the GC box did not bind any proteins in HepG2 nuclear extracts or compete for binding of nuclear proteins to the AF-1 or AF-2 oligonucleotides (data not shown). Oligonucleotides AF-1 and AF-2 also bound recombinant Sp1 (Promega; data not shown). However, nuclear extract from Bosc cells, a kidney cell line, and HepG2 cells had identical patterns of complex formation (data not shown).

Identification of novel sequences required for transcriptional activity of the FATP5 promoter

While the GC box between nucleotides 241 and 232 is essential for transcriptional activity, additional sequences downstream of the GC box might also be required for transcription. To determine if such sequences existed, we created 30 base pair internal deletions in the -271 construct downstream of the GC box. Constructs that had deletions in sequences between 240 and 180 nucleotides upstream of the FATP5

translational initiator had greatly reduced transcriptional activity in HepG2 cells (Fig 7). To identify the specific sequences within this region required for FATP5 transcription, a 10 nucleotide linker (CTAACAGGAG) was exchanged for wild type sequence within the context of the -271 base pair construct*. Transfection of these DNA's into HepG2 cells revealed two regions important for transcription. Mutating sequences between nucleotides -210 and -200 or between nucleotides -190 and -180 drastically reduced luciferase activity (Fig 8).

Discussion

In both humans and mice, FATP5 is only expressed in the liver. To determine the promoter elements mediating liver-specific transcription, we isolated a BAC encoding the mouse FATP5 genomic locus and sequenced 10 kb upstream of the transcriptional start. Since this 10 kb of genomic DNA did not contain either a TATA box or GC rich regions found in TATA-less promoters, FATP5 may utilize non-canonical sequences for transcription initiation. Unfortunately, attempts to identify the transcriptional start using primer extension were unsuccessful, perhaps due to secondary structure in the 5' UTR. Since we did not unambiguously determine the transcriptional start site, the nucleotide numbering in all of the promoter constructs refers to the distance from the translational start codon.

* Inadvertently, the 210 to 200 construct had a single nucleotide insertion and the 190 to 180 construct had a two nucleotide insertion relative to the wild type sequence. However, several other linker constructs that also had equivalent insertions (230 to 220 or 170 to 160 for example) had high levels of luciferase activity. Thus the decrease in luciferase activity in the 190 to 180 and 210 to 200 constructs is due to changes in the nucleotide sequence and not the result of the nucleotide additions.

GC box and Sp1 transcription factors

Since another gene was situated approximately 8 kb upstream of the FATP5 initiator methionine, we hypothesized that promoter elements were likely within this region of DNA. A luciferase reporter construct containing this sequence was transcriptionally active in two liver cell lines but was inactive in cell lines derived from lung, muscle, kidney, skin, or fibroblasts. Deletion analysis of the -8 kb reporter construct revealed that the FATP5 promoter was contained within the 261 nucleotides upstream of the initiator methionine. Promoter activity in this -261 base pair piece required the presence of a single GC box. Gel shift assays with oligonucleotides containing this GC box revealed the presence of three distinct complexes that required a functional GC box for binding. GC boxes bind the Sp1 family of transcription factors and the multiple complexes could reflect the binding of different members of the Sp1 protein family or different post-translational modifications of Sp1 in HepG2 cells (Rodenburg et al., 1997). Although the Sp1 family of transcription factors is widely expressed, Sp1 has been shown to be important for the transcription of several liver-specific genes and is upregulated in liver after birth (Rodenburg et al., 1997). In some cases, Sp1 will facilitate the binding of a tissue specific transcription factor to DNA. For example, Sp1 binding to DNA enhances the binding of C/EBP β to an adjacent site in the liver-specific CYP2D5 promoter (Lee et al., 1994). Since the C/EBP β binding site in the CYP2D5 promoter is suboptimal, C/EBP β binding to this site requires the presence of Sp1 or nuclear extract. A similar situation could occur in the FATP5 promoter. Although mutations in the 10 nucleotides downstream of the GC box had no effect on luciferase activity, we did not test mutations immediately upstream of the GC box for effects on promoter activity. It is also possible that Sp1 might bind an unknown liver-specific transcription factor and recruit it to the

FATP5 promoter. Although, there is no experimental evidence for this, Sp1 has recently been shown to bind to a transcriptional activator so additional interacting proteins are possible (Ryu et al., 1999).

Other liver-specific transcription factors

Alternatively, since the Sp1 gene family is important for the transcription of many genes which are not liver specific, liver-specific promoter elements in the FATP5 promoter might be located elsewhere (Boisclair et al., 1993; Rongnoparut et al., 1991; Sorensen and Wintersberger, 1999). Analysis of the sequence downstream of the GC box using TFSearch (<http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html>) did not reveal any additional transcription factor binding sites of relevance (Heinemeyer et al., 1999; Heinemeyer et al., 1998). Furthermore, we were unable to visually identify binding sites for known liver-specific transcription factors in this sequence (De Simone and Cortese, 1992; Hanson and Reshef, 1997; Lai, 1992). Thus, we looked experimentally for additional promoter elements by mutating the sequence downstream of the GC box and identified two additional sites downstream of the GC box that were essential for FATP5 transcription. The sequences of these sites do not conform to any known transcription factor binding sites suggesting the either novel proteins bind these elements or that these elements bind known proteins in a novel manner. Preliminary gel shift data using oligonucleotides spanning these site suggests that these two elements may comprise a binding site for a single complex. Additional data suggests that the complex which binds to these two sites interacts with the GC box 30 base pairs upstream. Interestingly, we noted a palindromic sequence equally split between these two sites (Fig 9). Since many

transcription factors bind palindromic DNA elements, it is intriguing to speculate that these two sequences contribute to the binding site for a novel transcription factor. Current investigations are focused on identifying the proteins binding to these novel elements and how this element interacts with the GC box.

Several studies have shown that the FATP gene family is regulated by a variety of substances including LPS, cytokines, insulin, and diet (Frohnert et al., 1999; Hui et al., 1998; Memon et al., 1999). Especially intriguing has been a recent report that FATP1 is upregulated by PPAR α ligands in liver cell lines (Martin et al., 1997; Motojima et al., 1998). Since fatty acids may be endogenous activators of PPAR's, transcriptional regulation of FATP1 by PPAR's may represent a physiologic feedback loop (Gottlicher et al., 1992; Grimaldi et al., 1999; Schoonjans et al., 1996). Given that liver also expresses FATP5, it will be interesting to see whether this genes is also regulated by PPAR α and the tools developed here should help address this question.

Several factors make the FATP5 promoter amenable to further study. First, liver-specific transcription of FATP5 can be recapitulated using immortalized cell lines *in vitro*. Second, the minimal required promoter element that confers liver-specific transcription is very small. Third, transcriptional activity of this promoter is very robust. Thus, study of the FATP5 promoter may provide additional insight into the mechanisms of liver specific transcription and regulation of the FATP gene family.

Figure Legends

Figure 1 Northern analysis of hsFATP5

Three different northern blots from Clontech were hybridized with a probe from the 5' UTR of hsFATP5. PBL = peripheral blood lymphocyte.

Figure 2 8 kb of FATP5 genomic sequence is sufficient for liver specific transcription *in vitro*

A luciferase reporter construct containing 8 kb upstream of the FATP5 initiator methionine was transfected into various cell lines using calcium phosphate as described in Methods. 48 hours after transfection, luciferase activity was measured and normalized to β -galactosidase activity. For each cell line, fold induction was determined by dividing the relative luciferase activity of the 8 kb construct by that of the promoter-less luciferase reporter vector. The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM.

Figure 3 Deletion analysis of the FATP5 promoter

Constructs containing deletions of the FATP5 promoter were transfected into HepG2 cells, assayed for luciferase activity, and normalized to β -galactosidase (RLU). The labels on the vertical axis correspond to the length of the promoter segment as measured from the initiator methionine. The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM.

Figure 4 271 base pairs upstream of the FATP5 initiator methionine are sufficient for liver specific luciferase activity.

A luciferase reporter construct containing 271 base pairs upstream of the FATP5 initiator methionine was transfected into various cell lines using calcium phosphate as described in Methods. Forty eight hours after transfection, luciferase activity was measured and normalized to β -galactosidase activity. For each cell line, fold induction was determined by dividing the relative luciferase activity of the -271 base pair construct by that of the promoter-less luciferase reporter vector. The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM.

Figure 5 Mutations of the GC box abolish transcriptional activity

A. Schematic of mutations in the GC box aligned with the normal sequence. The GC box consensus sequence is underlined.

B. Constructs containing 271 base pairs upstream of the FATP5 initiator methionine with the mutations in the GC box depicted in part A were transfected into HepG2 cells, assayed for luciferase activity, and normalized to β -galactosidase (RLU). The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM.

Figure 6 Gel shift analysis of the GC box with HepG2 nuclear extracts.

A. Schematic showing the sequence of the oligonucleotides used in gel-shift studies.

The numbering reflects the distance from the initiator methionine. The two pairs of oligonucleotides are indicated by the lines and labeled AF-1 and AF-2.

B. End-labeled oligonucleotides were incubated with HepG2 nuclear lysate and run on a non-denaturing gel. The left panel indicates the results when the AF-1 oligonucleotides were used as the probe and the right panel when the AF-2 oligonucleotides were used as the probe. Competition studies included the addition of 100 fold molar excess of the same unlabeled oligonucleotide to the gel shift assay 10 min prior to the addition of the ³²P-DNA. The arrows indicate the specific complexes formed with the oligonucleotides and the free probe is labeled in the AF-2 experiment. The results shown are representative of an experiment performed at least three times.

Figure 7 30bp internal deletions of the FATP5 promoter identify another region required for luciferase activity in HepG2 cells.

Reporter constructs were transfected into HepG2 cells. Luciferase activity was measured and normalized to β -galactosidase activity (RLU). The labels on the horizontal axis correspond to the nucleotides that were deleted and the numbering is represents the distance from the initiator methionine. The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM. Note that the five fold higher RLU activity in this figure relative to figure 3 and 5 is the result of a manufacturer change in the β -galactosidase reagent.

Figure 8 Linker scan of the FATP5 promoter identifies two additional elements required for transcription in HepG2 cells. Reporter constructs were transfected into HepG2 cells.

Luciferase activity was measured and normalized to β -galactosidase activity (RLU). The labels on the horizontal axis correspond to the constructs in part A. The data shown represent the mean of three experiments done in triplicate. Error bars indicate the SEM. Please note that the lower RLU activity in this figure relative to figure 3 and 5 is also the result of a manufacturer change in the β -galactosidase reagent.

Figure 9 Schematic of the FATP5 promoter

The GC box and two motifs identified in the linker scan are boxed and labeled. An arrow indicates the translational initiator of the FATP5 protein. The two halves of the palindrome contained in the novel motifs and referred to in the discussion are underlined.

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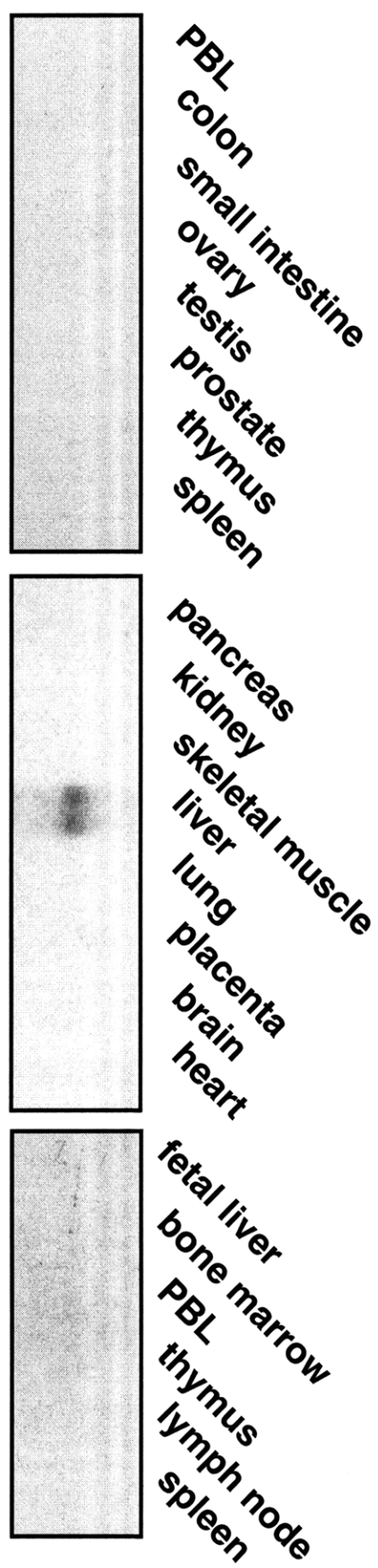
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Fig 1



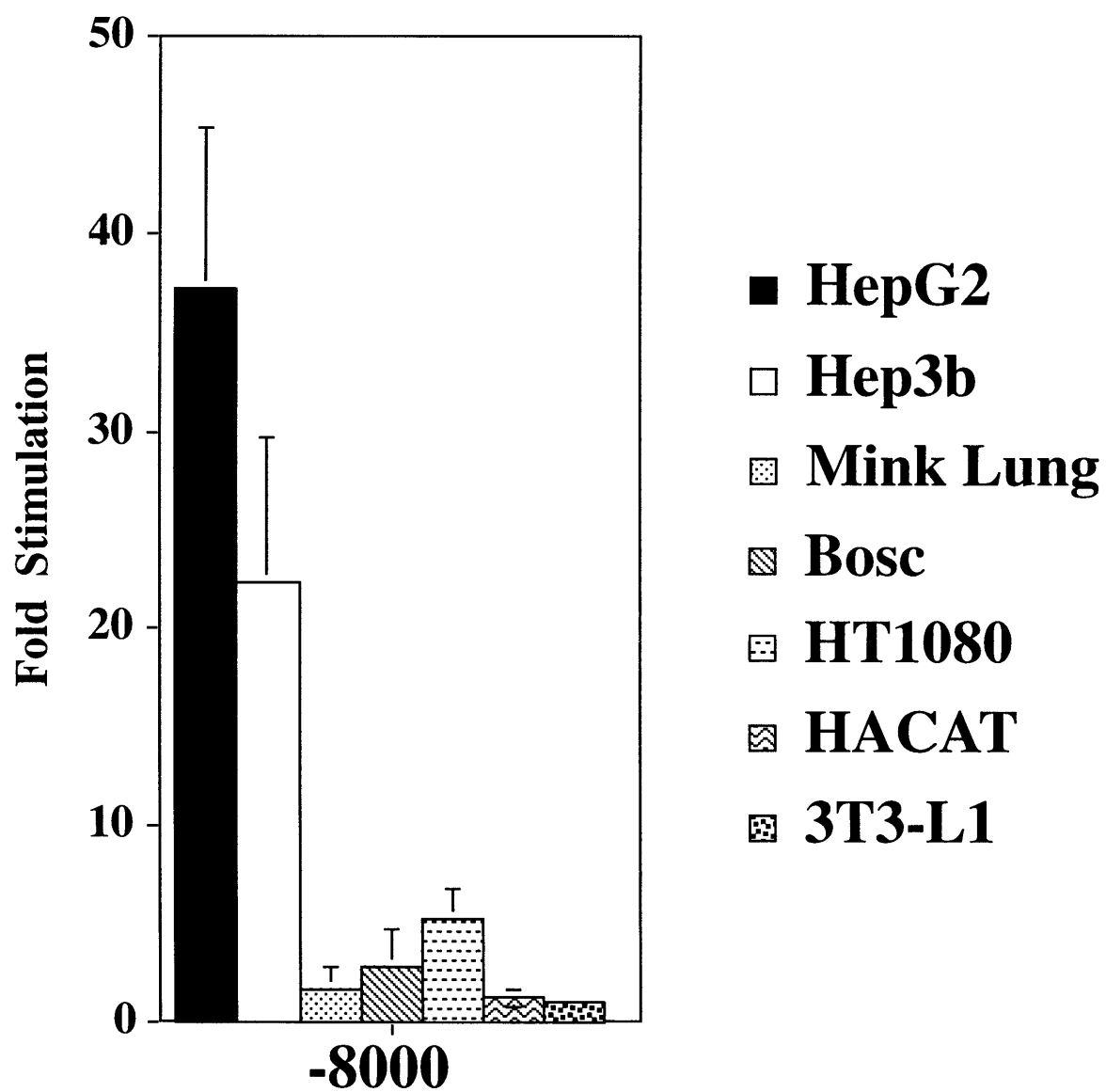


Fig 3

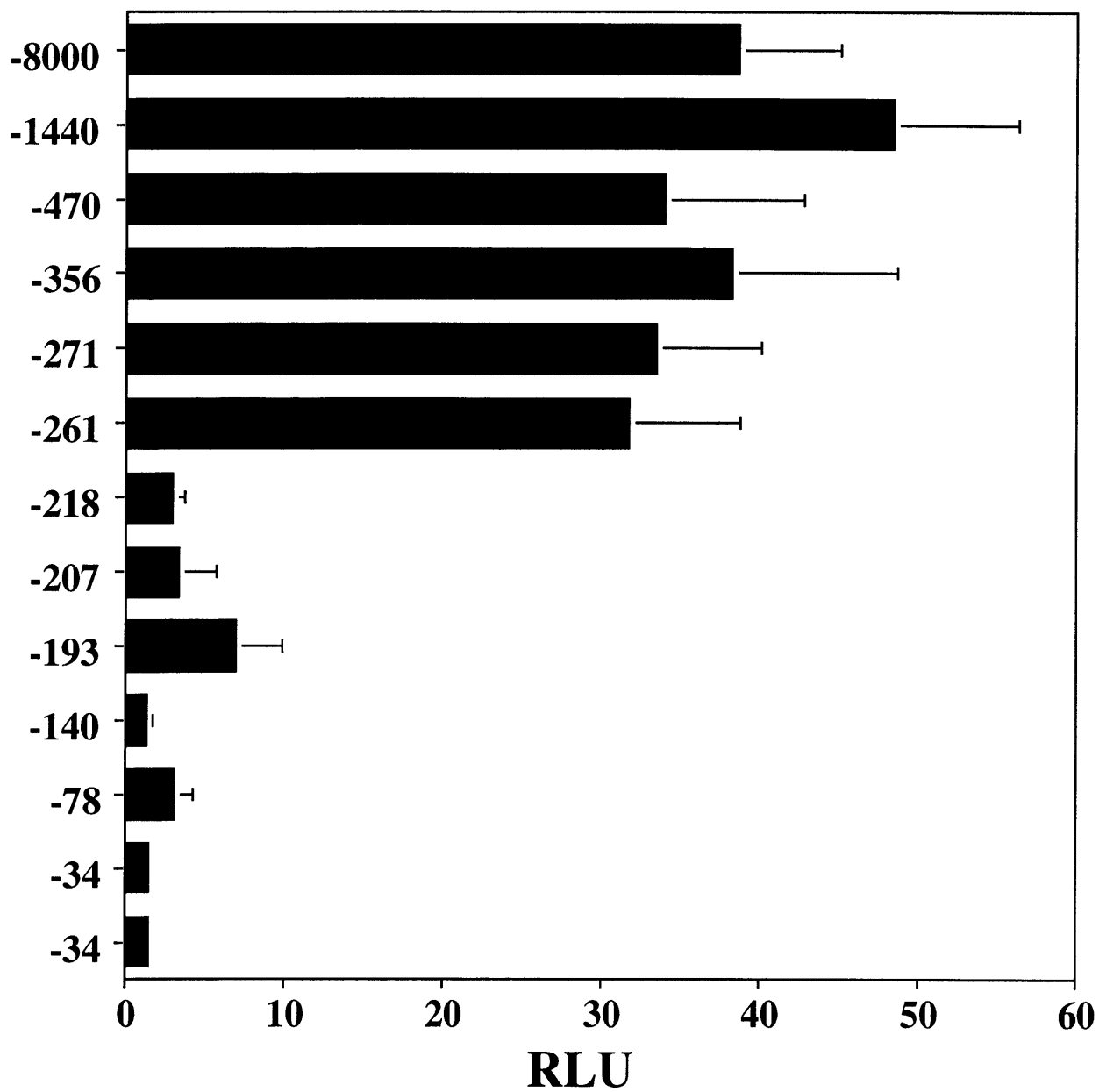


Fig 4

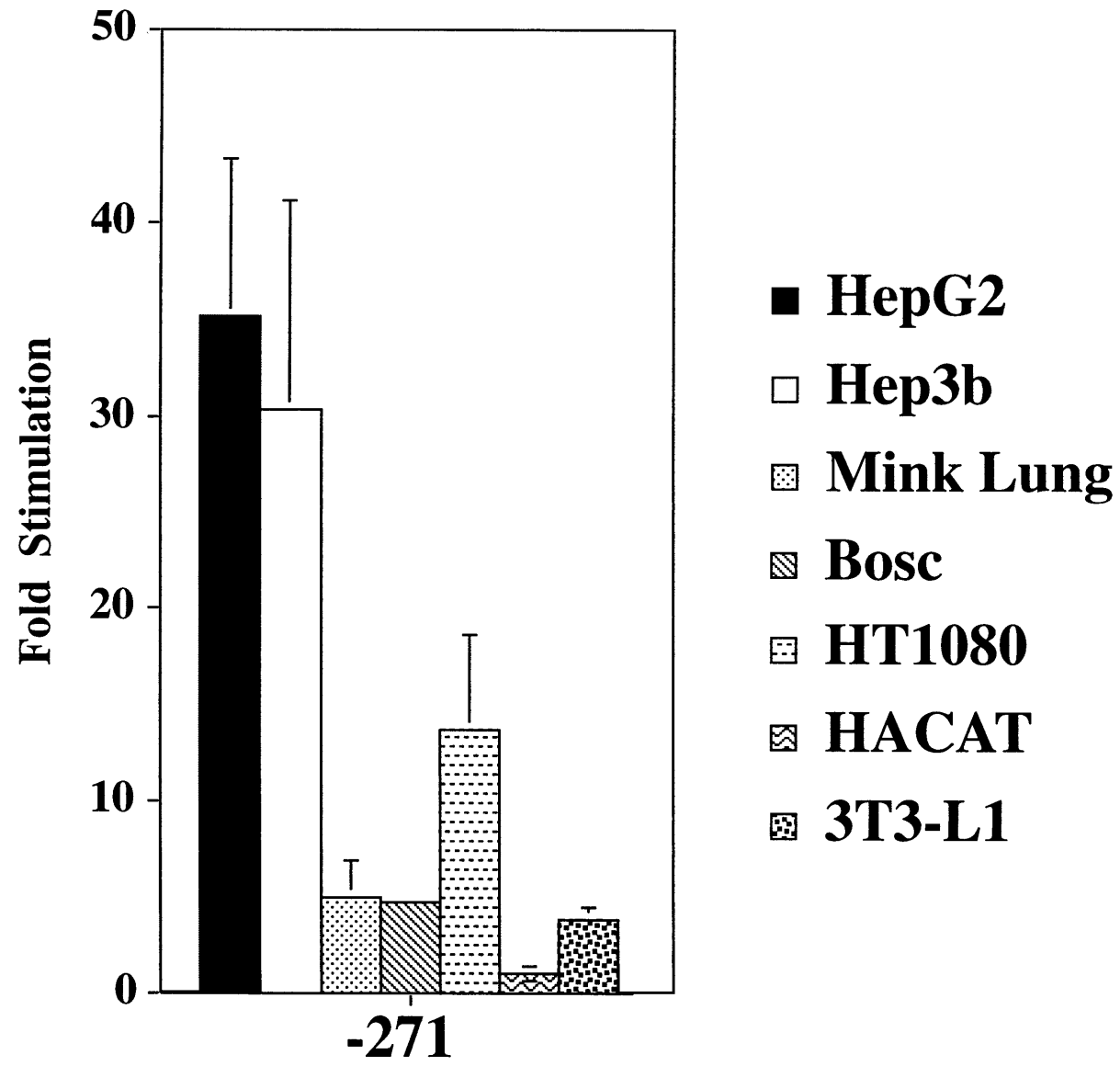
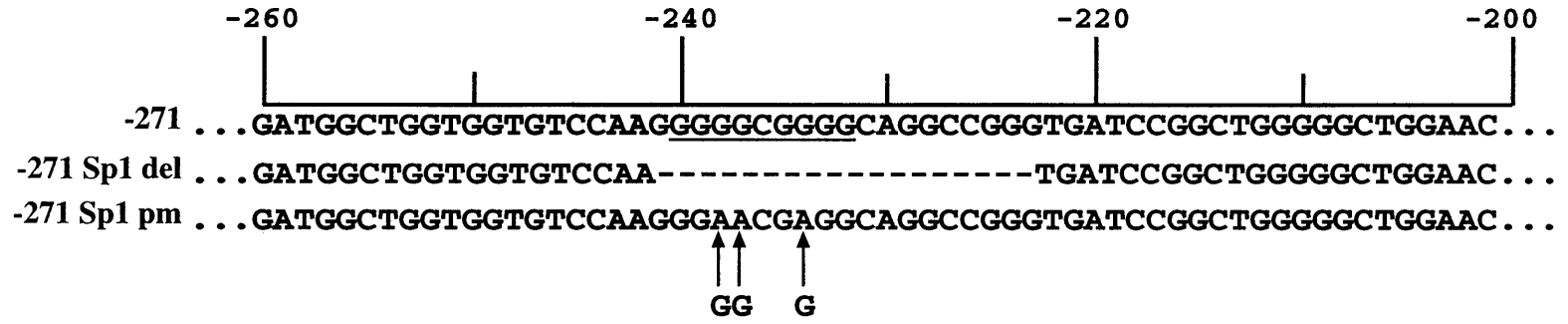


Fig 5

A.



B.

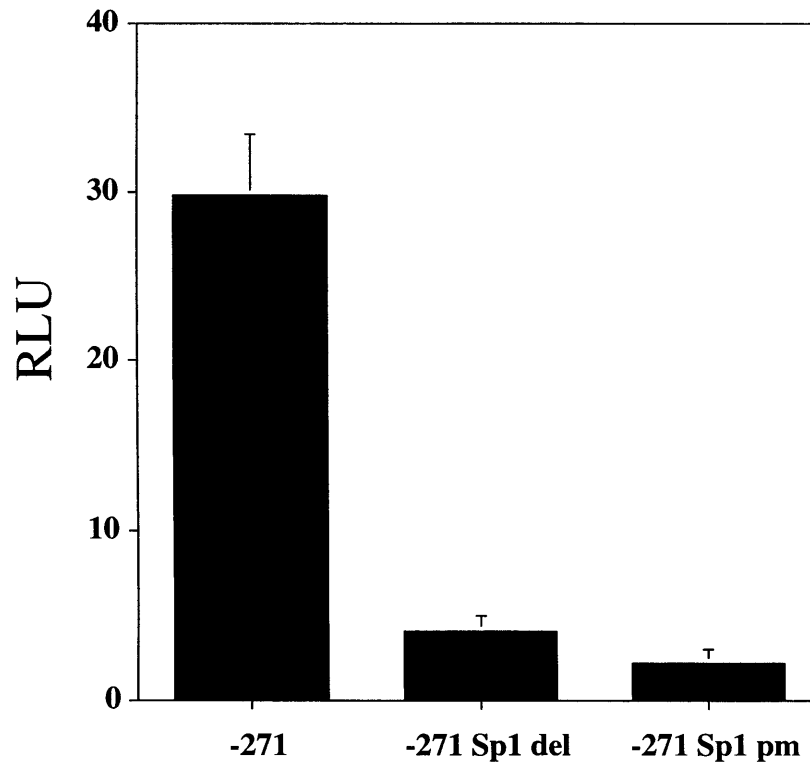


Fig 6

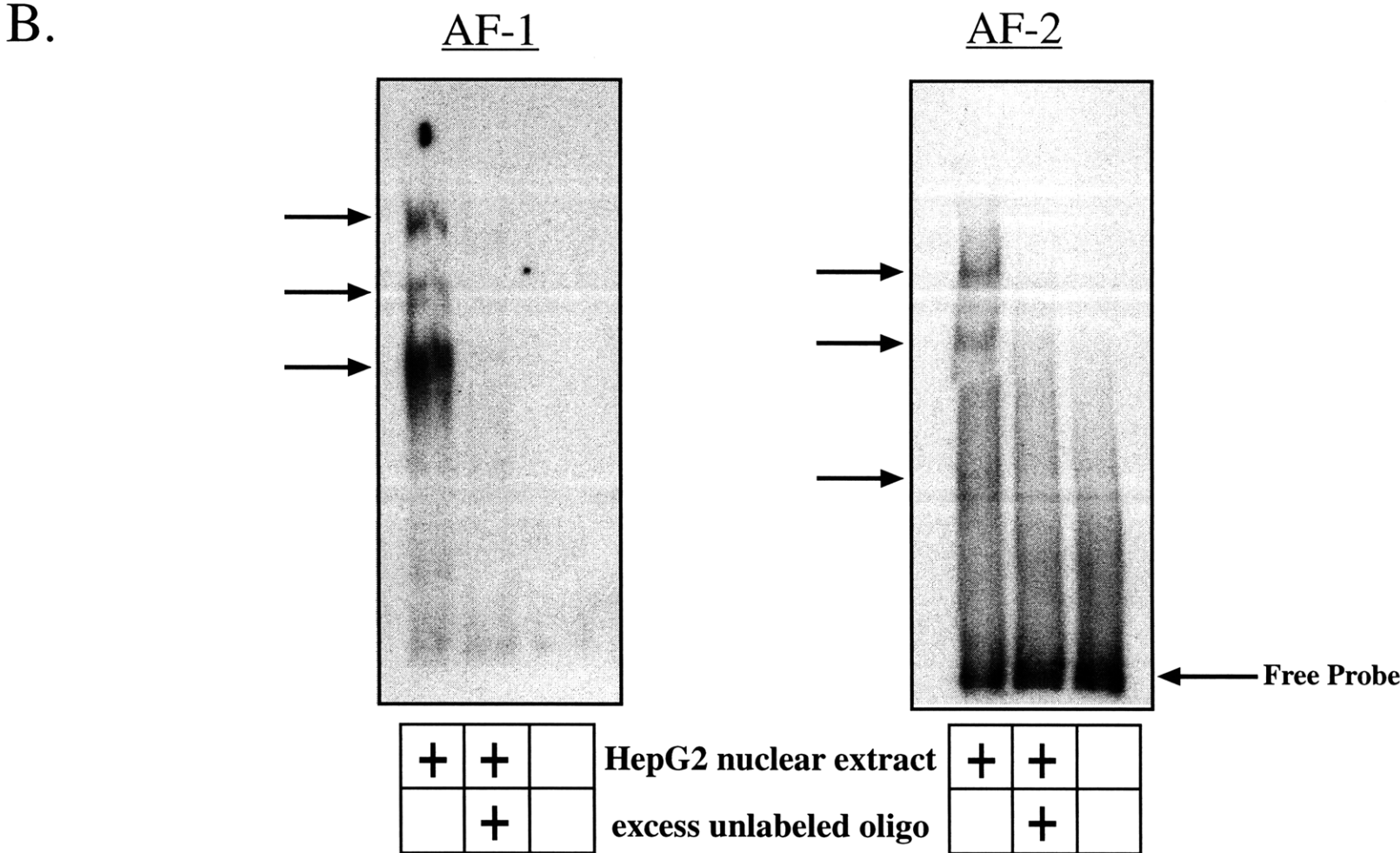
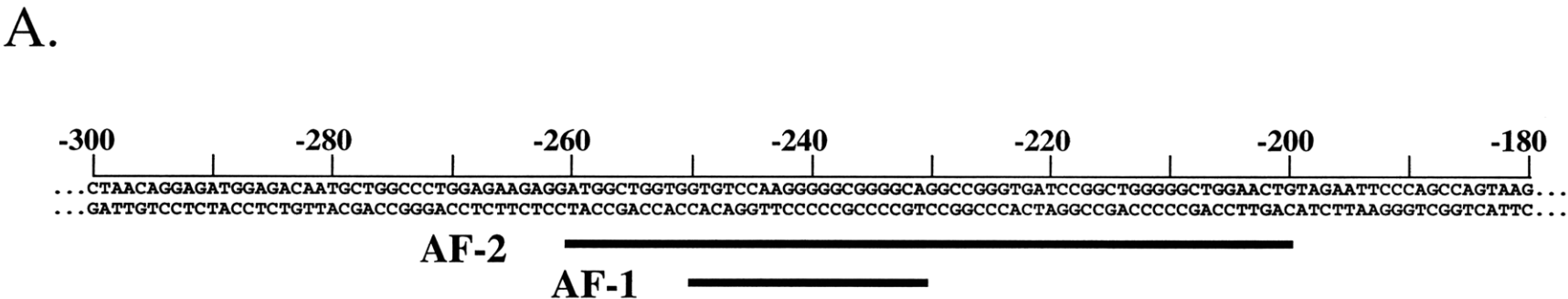


Fig 7

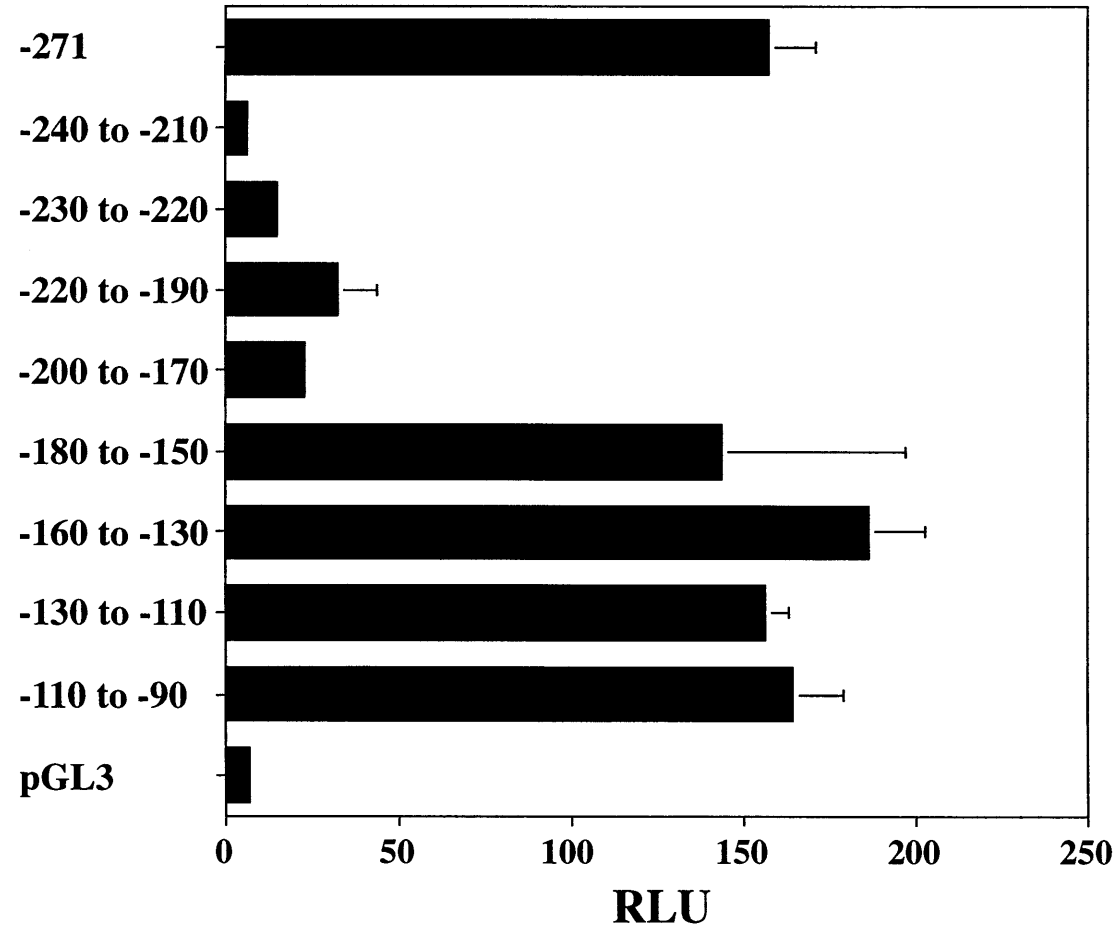


Fig 8

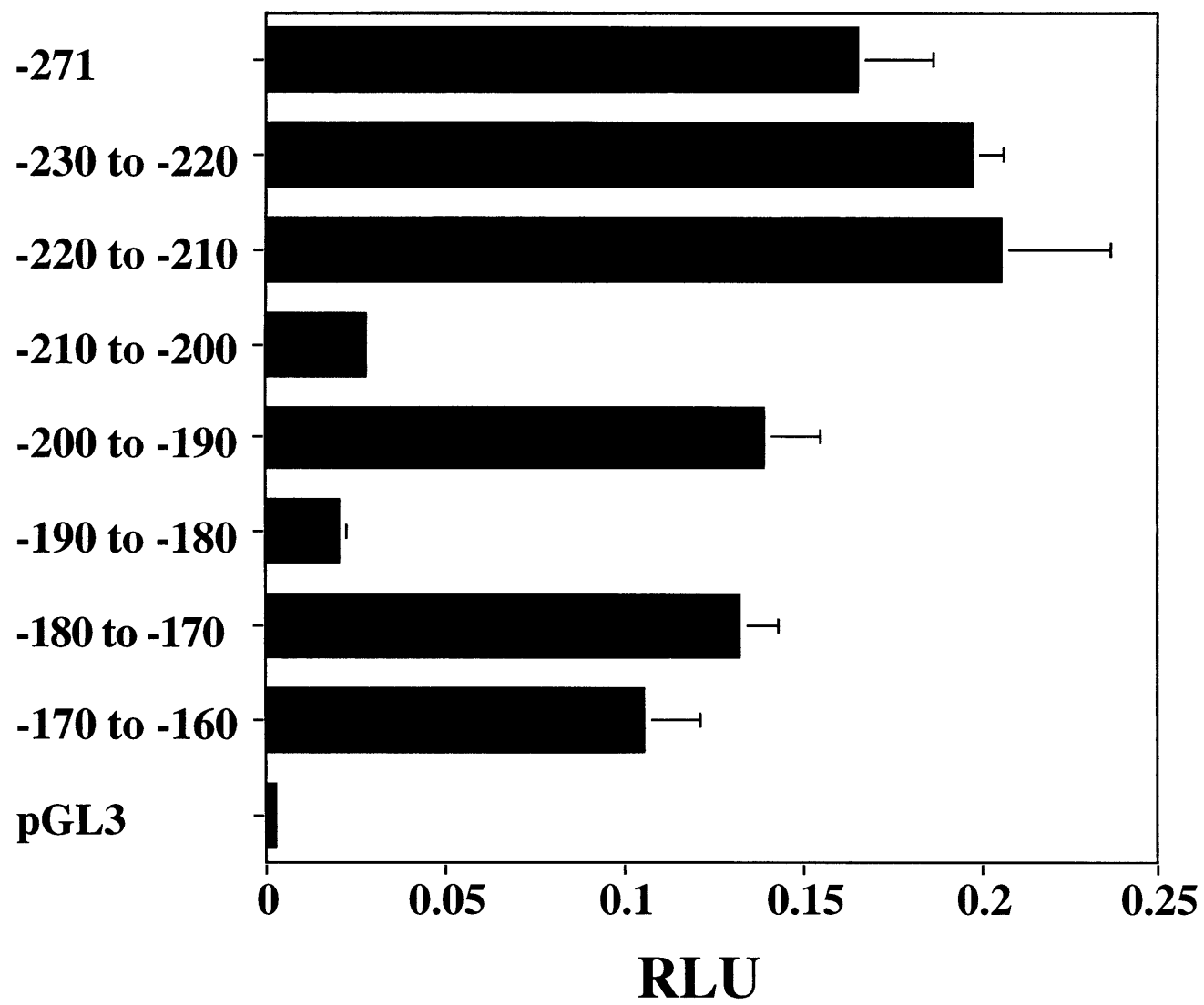


Fig 9

GAGGATGGCTGGTGGTGTCCAAGGGGGCGGGGCA
GGCCGGGTGATCCGGCTGGGGGCTGGAACTGTAG
AATTCCCAGCCAGTAAGAACTAAGTAACAAAAGG
ACAGAGTCCATGGGTCACATTCAGTTGCTGATAG
TACTTGGTCATATTTGGGAAGTGGGTAGACAGAT
TTCCTTAAAGGCAGGTAGTTAGGGCTTTGGAGCA
CTCATCAGAGCTAAGAGAGATTACACGCTCTCAT
CTACTTCAGAAAGAGCCAATGCCATG→

Sp1/GC box

Motif 1

Motif 2

Chapter 6

Concluding Thoughts

The successful evolution of humans required that many problems had to be solved and the solutions to these problems shaped human biology. Before the advent of the supermarket, the ability to store large amounts of energy was probably crucial for survival. Although energy can be stored as protein or carbohydrate, the primary reservoir for energy is fat stored as triglyceride in adipose tissue. An evolutionary biologist might ask why fat evolved as the vehicle for energy storage and several reasons are apparent. First, in contrast to glucose, the carbon atoms in fatty acids are not oxidized. Since ATP is produced by the oxidation of carbon, fat provides almost twice as much energy per carbon atom than glucose. Furthermore, fat can be stored at a much higher density than glucose or glycogen. Principally this is due to the hydroxyl groups on glucose which hydrogen bond with water molecules. Thus, glycogen *in vivo* contains significant amounts of water whereas triglyceride contains very little water.

However, the biochemical properties of fat that made it favorable for energy storage also presented certain problems that had to be solved. The key problem was how fat stored in adipocytes could be transferred to the organs which needed fatty acids for ATP production. Owing to their hydrophobic nature, fatty acids have an extremely low solubility in aqueous solution. This is partially compensated for in blood by high concentrations of albumin, which can bind fatty acids. Thus, although the free fatty acid concentration in serum is less than a hundred nanomolar, the total fatty acid concentration

in serum is several hundred micromolar when albumin-bound fatty acids are included. However, even with the albumin-bound reservoir of fatty acids, the concentration of fatty acids in serum is not high enough to meet the metabolic needs of tissues *in vivo*. Humans have therefore evolved another method to deliver fatty acids from adipocytes to the tissues. While some of the fatty acids released by adipocytes are immediately taken up by tissues for metabolism, the liver also takes up a large portion of these fatty acids. In the liver, fatty acids are re-esterified into triglyceride and packaged into lipoprotein particles which are essentially tiny lipid droplets with a protein coat. In fact, more than 90% of fat in serum is contained within lipoprotein particles. These lipoproteins are secreted by the liver and freely circulate throughout the body. Fatty acids are liberated from lipoprotein particles by lipases which are found in the capillaries of metabolically active tissues. So, the adipocyte and hepatocyte work in conjunction to turn the large, immobile lipid droplet of the adipocyte into tiny, freely circulating lipid droplets.

During times of starvation, the liver and adipocyte work together to deliver nutrients throughout the body. However, when food is plentiful, fatty acid flux in the adipocyte is reversed. Instead of releasing fatty acids into circulation, adipocytes take up fatty acids and turn them into triglyceride for storage. The signals that regulate adipocyte fatty acid flux are hormones such as insulin and glucagon which vary according to the nutritional state of an individual. The effect of these hormones on fatty acid transport in the adipocyte was the focus of chapter 2. Insulin, which rises after a meal and energy is plentiful, stimulates fatty acid uptake. Glucagon, which rises in-between meals when energy supplies are low, stimulates fatty acid efflux. Unlike insulin and glucagon, increases in epinephrine, which stimulates fatty acid efflux, are not linked to nutritional status. Epinephrine is released during times of stress (i.e. fleeing the proverbial saber toothed tiger) when an individual would seek to mobilize as much energy as possible

regardless of nutritional status. While these hormones normally regulate fatty acid flux, the TNF α inhibition of fatty acid uptake described in chapter 2 likely reflects a pathologic state. Hotamisligil et al. have shown that increased production of TNF α is causally linked to elevated levels of serum free fatty acids in animal models of obesity and diabetes (Hotamisligil et al., 1993). High concentrations of serum fatty acids inhibit the ability of insulin to shut down hepatic glucose output after meals which leads to hyperglycemia (Lewis et al., 1997). Thus, the data in chapter 2 would suggest that TNF α inhibition of adipocyte fatty acid uptake might be one of the early steps in the evolution of diabetes. Interestingly, Uysal et al. have shown that knocking out the TNF α gene in a mouse model of diabetes lowers circulating free fatty acids and improves insulin sensitivity (Uysal et al., 1997).

Regulated movement of fatty acids into and out of adipocytes requires effector molecules downstream of the hormones. In the case of glucagon and epinephrine, fatty acid efflux is due to the activation of hormone-sensitive lipase by protein kinase A phosphorylation. On the other hand, regulation of fatty acid uptake by insulin and TNF α is only partially understood. Although signal transduction cascades for these hormones have been studied in great detail, all of the steps between hormone binding to a receptor and alterations in fatty acid flux are not known. In addition to fatty acid transport, these hormones regulate glucose uptake in adipocytes by altering the number of glucose transporters on the cell surface. That is, insulin stimulates glucose uptake by increasing the number of glucose transporters on the cell surface while TNF α inhibits glucose uptake by inhibiting transcription of glucose transporters (Stephens et al., 1997). If an analogy were to be drawn with glucose uptake, insulin and TNF α alterations in fatty acid uptake could be due to the regulation of a fatty acid transporter.

In fact, six different plasma membrane transporters have been identified and four of these increase fatty acid uptake when over-expressed in cell lines. Although FABPpm, CD36, and ADRP increase fatty acid uptake *in vitro*, several reasons suggest that these proteins are unlikely to mediate fatty acid transport *in vivo* (reviewed in chapter 1). On the other hand, the data in chapter 4 demonstrate that a member of the FATP gene family, FATP4, may be responsible for fatty acid uptake by intestinal epithelial cells *in vivo*. However, the only way to unequivocally establish that FATPs mediate fatty acid transport *in vivo* will be to generate knockout mice for these genes. A clear prediction from the data in chapter 4 is that FATP4 knockout mice should be unable to absorb dietary fatty acids. Although the liver expresses two FATPs at high levels, another prediction would be that hepatocytes in a FATP2 and FATP5 double knockout should also have a defect in fatty acid uptake. Fortunately, all of the FATPs are unlinked (unpublished data) so crossing the single knockouts with each other can easily yield the double knockouts. In addition to the knockouts, the transcriptional regulation of these genes may also yield additional insight into their function. For instance, the clinical efficacy of the antidiabetic thiazolidinediones might be due to upregulation of FATP1 mRNA in adipocytes (Martin et al., 1997). That is, FATP1 upregulation could increase adipocyte fatty acid uptake which would then lower serum free fatty acids and improve insulin sensitivity. Similarly, the FATP5 promoter described in chapter 5 may provide insight into the regulation of hepatocyte fatty acid uptake.

The discovery of the Glut family of glucose transporters was instrumental in understanding the physiologic regulation of glucose fluxes *in vivo*. Discovery of the FATP gene family may provide similar insight into fatty acid transport.

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Dedicated to three great women in my life...

my grandmother, Eva Mendelson,

my mother, Peggy Hirsch,

my future wife, Amy Fallon.